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INCREASED RECOMBINATION FROM FEMALE DROSOPHILA IRRADIATED AS LARVAE WITHOUT OÖCYTES

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Received January 16, 1961

VARIABILITY in linkage data has been characteristic of studies on crossing over in Drosophila, and it remains unexplained. GOWEN (1919) has shown that the recombination fraction from female to female varies more than do many physical characteristics. It has subsequently been presumed that such changes resulted from fluctuating amounts of crossing over during meiosis, but this correspondence has not been rigidly demonstrated. Another cell stage, the gonia, should also be considered. In Drosophila males, normally without crossing over, numerous experiments show clearly that crossovers originate in spermatogonial cells after irradiation (WHITTINGHILL 1955) or after chemical mutagen treatment (WHITTINGHILL and LEWIS 1961). One indication of gonial origin is the tendency to recover unequal numbers of noncrossovers—uneven in a predictable direction—along with grossly unequal complementary classes of the crossovers. Currently the question is whether induced gonial exchange occurs in normal female Drosophila.

MATERIALS AND METHODS

Methods standard for Drosophila were employed. Cultures of yeasted corn meal molasses-agar were maintained at 24–26°C. Recessive stocks of "rucuca" (elaborated in Table 1), of vestigial, *vg*, and of *vg* rucuca were used. [rucuca = third chromosome multiple stock, includes *ru* (0.0) *h* (26.5) *th* (43.2) *st* (44.0) *cu* (50.0) *sr* (62.0) e^s (70.7) *ca* (100.7).] Mass matings between *vg* rucuca females and *vg* males were made in an attempt to reduce the number of origins of gonial cells in the F_1 by having them homozygous for vestigial, which has among its pleiotropic effects a reduction in the number of egg strings (ovarioles). Eggs were collected for six hours on a food cake on a polyethylene cap for future irradiation *in situ*, and the larvae grew in a ventilated lucite vial. Cultures having masses of larvae about to pupate were selected to be X-rayed or to accompany treatment material to the X-ray room and to share in any slight temperature changes. The X-raying was done at the N. C. Memorial Hospital with a Picker machine operating at 140 kv, 20mA with inherent filtration of .25 mm Cu and 1 mm Al. Irradiation at 36 cm target distance for 7 + 10 + 11 minutes provided doses approximating 1000r, 2500r and 4000r.

Upon emergence *vg* females (homozygous *vg*, heterozygous rucuca) from the

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TABLE 1

Regional crossover frequencies in percent ± S. E. in Brood 1 (days 1–4) from F_1 vg/vg; *rucuca/ + females and* ru h th st cu sr e^s ca *males*

Group	Regions 1	2	3*	4	5	6	7	Total offspring
Control	19.2 ± 2.2	13.3 ± 1.9	..	1.2 ± 0.6	5.3 ± 1.2	6.5 ± 1.4	37.8 ± 2.7	323
1000r	24.5 ± 2.5	15.0 ± 2.0	..	2.9 ± 1.0	13.1 ± 1.9	6.5 ± 1.4	38.9 ± 2.8	306
2500r	19.8 ± 2.8	20.8 ± 2.9	..	10.2 ± 2.2	14.7 ± 2.5	6.1 ± 1.7	33.5 ± 3.4	197
4000r	18.4 ± 5.8	31.8 ± 7.0	..	9.1 ± 4.3	6.8 ± 3.8	6.8 ± 3.8	38.6 ± 7.3	44
								P = .005

* Region 3 had one crossover each in the control, 1000r and 4000r groups.

three treatment groups and from controls were coded and testcrossed individually to several rucuca males and were allowed to lay eggs in five successive bottle cultures starting 1, 5, 8, 11 and 14 days after eclosion. Classification of the offspring was made only during the eight days following the first emergences in each culture. The coded data were rearranged into age and treatment groups for statistical analyses only after the offspring from the final transfers had been classified. Chi-square tests indicated homogeneity within each treatment group.

RESULTS

Induced crossovers were found only in the first two of the five broods upon testing the three treatment and one control groups per brood. Regional crossover frequencies for Brood 1 are presented in Table 1. A higher frequency of crossovers was found in the more central regions of irradiated chromosomes at this time, i.e., Regions 2, 3, 4 and 5, but not all of the increases above control frequencies were individually significant. Homogeneity x^2 was 71 for this brood meaning a P value of 0.005, due chiefly to control noncrossovers being low and to contrasts between control and treated groups in Regions 4 and 5.

The effect of irradiation wore off in subsequent broods. In Brood 2 the homogeneity x^2 was 35, giving a P value of 0.023. Broods 3, 4 and 5 (8–21 days) were homogeneous as tested by region of the chromosome and irradiation dose with P values of 0.52, 0.91 and 0.42, respectively. This later similarity indicated that there were no apparent chromosomal differences between females chosen for the different treatment categories, and this uniformity was confirmed when the data for all broods were added within treatment groups and controls. Therefore differences in Brood 1 and in Brood 2 may safely be attributed to the treatments which the larvae had received.

The crossover changes could be checked by examination of the noncrossover figures, which, on the simplest assumption, should vary inversely. All treatment groups were below the control in frequency of parental types in Brood 1 (Table 2), and the 1000r group was below the control and all others in Brood 2 by a statistically significant amount.

TABLE 2

Noncrossover frequencies in percent with standard errors by broods

Group	Broods 1 1–4 days	2 5–7 days	3 8–10 days	4 11–13 days	5 14–21 days	Mean noncrossovers
Control	37.8 ± 2.7	33.6 ± 4.4	27.6 ± 4.0	27.4 ± 4.0	24.6 ± 3.8	31.8 ± 1.64
1000r	25.8 ± 2.5	11.9 ± 5.0	60.0 ± 22.0	32.4 ± 4.0	24.1 ± 2.7	25.9 ± 1.58
2500r	27.4 ± 3.2	39.5 ± 3.8	33.7 ± 5.1	30.8 ± 7.4	23.3 ± 4.2	31.3 ± 1.91
4000r	20.4 ± 6.7	40.3 ± 6.0	40.0 ± 7.3	26.6 ± 5.0	28.8 ± 3.6	30.9 ± 2.32

DISCUSSION

Although the irradiation was given early, and although increased total crossovers were evident, recognizable inequalities of complementary crossovers were not found, nor did the two kinds of noncrossovers show any irregularities. It was hoped that a reduction in the number of egg strings would reveal some induced mosaicism among the remaining egg strings, but the dissection of *vg* rucuca stock females revealed 11 ovarioles per ovary, which was a very slight reduction from the 15 egg strings per ovary of ordinary females. The presence of so many ovarioles would still permit the occurrence of many instances of mitotic recombination with individually small upsets of the ratios between classes but with cumulatively large effects upon mean recombination frequencies. In each egg string one or more of the older gonia might have undergone mitotic crossing over while still younger gonia, in this experiment, showed no detectible effect upon the eggs eventually laid in the last three broods.

The study of the noncrossovers was made to determine whether there were appreciable numbers of radioresistant cells, or crossover resistant cells among susceptible cells. If so, noncrossover chromosomes might be found uniformly even in broods where single crossovers were preferentially becoming doubles and doubles becoming multiple crossovers to yield high recombination when regions were totalled. Such was not the case. The low frequency of the Brood 2 noncrossovers following 1000r was in the opposite direction from what could be expected if the population of irradiated gonia contained some cells which were more radioresistant than their neighbors. It is also opposite to the frequency expected if there was any considerable weeding out of injured cells in this experiment.

The unexpected higher frequency of crossovers from older control females was confirmed by the distribution of noncrossovers among the same families and also by the noncrossovers in the last three broods of the irradiated series, i.e., in Table 2 the noncrossovers in each Brood 5 are below the mean for all broods of that treatment or control group. Thus the crossover maximum was in Brood 5 rather than, as observed by BRIDGES (1927), in Brood 1.

Increased crossovers from Drosophila irradiated as larvae contrast sharply with the failure of any induction from irradiated Habrobracon (WHITTINGHILL and ALLEN 1961). In both experiments irradiation was timed to precede the formation of any oöcytes (KERKIS 1931). Possible conclusions depend upon one's

assumptions *in re* the cell generation in which induced crossing over is completed. If the action of X-rays is assumed to be delayed until meiosis begins, a difference between the maturation processes in the two insects would be appropriately concluded. If, however, the X-rays acted directly, one would infer a difference in radiosensitivity of their oögonia. Because male Drosophila complete crossing over in gonia (WHITTINGHILL 1955), we believe that the action upon female crossing over was direct and that the basic oögonial difference is the somatic pairing of chromosomes in Diptera and the absence of such association of chromosomes during mitosis in Habrobracon.

SUMMARY

1. Different X-ray doses to late third instar larvae of Drosophila (heterozygous for the rucuca recessives and homozygous for vestigial) produced elevated frequencies of crossovers only from eggs laid in the first week of the imagoes. Three broods from 8–21 day-old adults were homogeneous among control and three irradiation dosage groups.

2. Comparison of noncrossover and crossover frequencies showed no evidence of radioresistant cells among the oögonia present in X-rayed larvae.

3. Higher crossover and lower noncrossover values were found in the 14–21 day brood rather than in the earliest brood of the controls.

4. This response of Drosophila and the lack of such response of Habrobracon to irradiation are discussed.

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CYTOGENETIC ANALYSIS OF THE ACTION OF CARCINOGENS AND TUMOUR INHIBITORS IN DROSOPHILA MELANOGASTER. IX. THE CELL-STAGE RESPONSE OF THE MALE GERM LINE TO THE MESYLOXY ESTERS

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A STUDY of the mutagenicity of a series of nitrogen mustards has shown that the structure of the nonalkylating (or prosthetic) moiety of the molecule has some bearing on biological activity. The same molar dose of difunctional compounds with roughly equal chemical reactivity, but with different prosthetic groups, induced in the sperm significantly different mutation rates (FAHMY and FAHMY 1960a). The prosthetic group of the mustard molecule also played a major role in the determination of the pattern of cell-stage response during spermatogenesis. The relative mutation rate of the various stages of the male germ line was markedly different according to whether the mustard was a derivative of an amino acid, a carboxylic acid, or an amine (FAHMY and FAHMY 1960b).

Another series of the alkylating agents which proved to be effective mutagens on the testis of adult Drosophila, is the mesyloxy esters (BIRD 1951; FAHMY and FAHMY 1956a). The mutagenic cell-stage response during spermatogenesis under a homologous series of monofunctional methanesulphonates was investigated and proved to be fairly consistent and characteristic: the spermatogonial stages were refractory, while the postspermatogonial stages were responsive, with maximal effect on mature sperm (FAHMY and FAHMY 1957). The question then arose as to whether this pattern of cell-stage response is typical of the mesyloxy esters. The analysis of the cell-stage effect was accordingly extended to several other sulphonates with a view to the elucidation of the consequences of changes in the mutagenic molecule, both as regards the number of the functional groups (the mesyloxy radicals) and the structure of the prosthetic moiety.

MATERIAL AND TECHNIQUE

The compounds analysed are all esters of methanesulphonic acid, and were synthesized in the chemistry department in relation to cancer chemotherapy (HADDOW 1955). From the chemical point of view they are either mono- or difunctional as regards the alkylating groups (the mesyloxy radicals) but with different prosthetic moieties; the latter varied as regards chain length, valency unsaturation, steric configuration, and degree of hydroxylation (substitution by

-OH groups). The code numbers, structural formulae and chemical names of the compounds tested will be here listed under their appropriate chemical subseries. For ease of reference each subseries will also be given an abbreviated name.

Alkyl methanesulphonates: 'Monomesylates' $R\text{-}OSO_2CH_3$

CB.1540: $R = CH_3$; methyl methanesulphonate.

CB.1528: $R = C_2H_5$; ethyl methanesulphonate.

Dimethanesulphonoxyalkanes: 'Dimesylates' $CH_3O_2SO\text{-}R\text{-}OSO_2CH_3$

CB.2040: $R = (CH_2)_3$; 1:3-dimethanesulphonoxypropane

CB.2041: $R = (CH_2)_4$; 1:4-dimethanesulphonoxybutane (Myleran)

CB.2058: $R = CH_2C \equiv C\,CH_2$; 1:4-dimethanesulphonoxybut-2-yne

CB.2094, CB.2095: $R = CH_2CH = CHCH_2$ { *cis* and *trans* isomers of 1:4-dimethanesulphonoxybut-2-ene

Dimethanesulphonylalkanols: 'Polyol-mesylates'
$CH_3O_2SOCH_2(CHOH)_nCH_2OSO_2CH_3$.

CB.2511, CB.2628: n = 4; D and L-isomers of 1:6-dimethanesulphonyl mannitol.

CB.2562: n = 2; 1:4-dimethanesulphonyl erythritol.

All compounds were administered as aqueous solutions in isotonic saline (0.4 percent NaCl) by microinjection around and within the testes of adult flies of the Oregon-K stock (details in FAHMY and FAHMY 1960a). The mono- and polyol-mesylates offered no difficulty as regards solubility; but the dimesylates, especially the higher homologues (CB.2041, 2058, 2094 and 2095), were sparingly soluble. By vigorous grinding during dissolution and a slight raising of the temperature (where necessary) it was possible to solubilize most of these compounds for the biological tests. The exceptions were the butane (CB.2041) and the butyne (CB.2058) derivatives with maximal solubility of 0.23 and 0.41×10^{-2}M, respectively. Higher doses were administered as partial suspensions.

The mutations analysed are the sex-linked recessive lethals as detected by the Muller-5 technique. The stock used for the genetic tests has been kept in our laboratory for many years and gives a spontaneous sex-linked recessive lethal rate of 0.18 ± 0.05 percent. The progeny of the treated males was fractionated according to our standard brood technique (FAHMY and FAHMY 1954, 1955, 1960b). The males were treated at 30±5 hours after eclosion and were repeatedly mated to virgin females at three-day intervals. Breeding was undertaken in mass cultures with an approximately equal male to female ratio. This mating procedure gives essentially the same brood-mutation pattern as pair cultures (FAHMY and FAHMY 1955, 1960c). It has the advantage of enabling the recovery of progeny from a large number of treated males, thus minimizing the effect of variability of mutagenic response between individuals.

OBSERVATIONS

Alkyl methanesulphonates: The brood-mutation curves for representatives of the monomesylates are illustrated in Figure 1, and the experimental data are given in Table 1. There is clearly a trend towards a lower mutation rate the

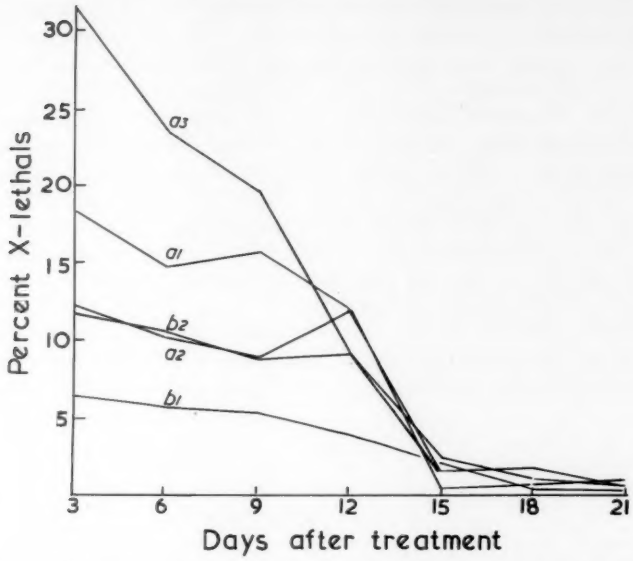

FIGURE 1.—The brood-mutation curves for the monomesylates. Curves lettered to correspond with experiments in Table 1.

TABLE 1

Sex-linked recessive lethals induced in the successive broods (three-day intervals) by various monomesylates

	CB.1528						CB.1540			
	a1		a2		a3		b1		b2	
Brood	1.61×10^{-2}M chr.	%1	2.42×10^{-2}M chr.	%1	9.18×10^{-2}M chr.	%1	0.25×10^{-2}M chr.	%1	0.45×10^{-2}M chr.	%1
I	458	18.3	516	12.2	70	31.4	509	6.3	455	11.6
II	350	14.6	547	10.2	51	23.5	473	5.7	506	10.5
III	301	15.6	537	8.8	46	19.6	504	5.2	677	8.7
IV	383	12.0	537	11.9	44	9.1	487	3.9	482	9.1
V	316	0.3	537	1.5	144	1.4	481	2.1	494	2.4
VI	345	0.6	541	1.7	..	..	576	0.3	493	1.0
VII	328	0.9	547	0.5	..	..	511	0.2	326	0.6
Total	2481	9.4	3762	6.6	355	13.8	3541	3.3	3433	6.6

later is the brood, the fall being most drastic between the fourth and fifth broods. The mutation rate in the latest broods in all experiments is of the same order as, or slightly above, the spontaneous rate.

The statistical significance of the fluctuations in the mutation rate between broods was undertaken according to YATES' (1955) technique. The relative fre-

quency of lethal to nonlethal chromosomes for each pair of consecutive broods in the same experiment is compared in a 2×2 contingency table, and x for the deviation is computed with sign: negative for a drop, and positive for a rise in the mutation rate. The algebraic sum of x for the same pair of broods in different experiments is a normal deviate with a standard deviation $= \sqrt{n}$, where n is the number of experiments. The data for the separate experiments with the individual compounds, as well as the combination test for all the monomesylates, reveals a decisive brood-mutation pattern (Table 4A). The mutation rate drops consistently from the first to the second brood and continues to decrease in the majority of the experiments till the fourth brood. A further gross depression then occurs between the fourth and fifth broods, and the very low rate reached is maintained until the end of fractionation.

Dimethanesulphonoxyalkanes: The brood-mutation relationship for a homologous series of dimesylates is given in Table 2 and Figure 2. The low solubility and high toxicity of the majority of these compounds did not allow a wide range

TABLE 2

Sex-linked recessive lethals induced in the successive broods (three-day intervals) by various dimesylates

	CB.2040							
	c1 0.9×10^{-2}M		c2 3.0×10^{-2}M		c3 4.7×10^{-2}M		c4 9.5×10^{-2}M	
Brood	chr.	%1	chr.	%1	chr.	%1	chr.	%1
I	482	3.5	365	9.9	406	11.8	351	10.5
II			382	9.7	266	10.5		
III	480	2.7	264	8.0	198	8.6	264	8.7
IV	14	..	191	4.2	86	7.0	9	..
Total	976	3.1	1262	8.5	956	10.4	624	9.6
	CB.2041 d 0.6×10^{-2}M		e1 0.2×10^{-2}M		CB.2058 e2 0.4×10^{-2}M		e3 0.4×10^{-2}M	
I	245	2.9	442	2.0	286	6.3	586	4.6
II	172	1.7	427	1.6	261	4.6	496	4.0
III	..	..	438	0.5	..	..	..	..
IV	..	..	421	0.0	..	..	..	..
Total	417	2.4	1728	1.0	547	5.5	1082	4.3
	CB.2094 f 0.25×10^{-2}M		g1 0.25×10^{-2}M		CB.2095 g2 0.4×10^{-2}M		g3 0.7×10^{-2}M	
I	516	0.6	486	2.1	475	2.5	384	4.4
II					439	1.4	121	2.8
III	480	0.2	469	1.1	444	0.9	..	..
IV	412	0.2	419	0.5	420	0.5	..	..
V	..	..	..	..	430	0.2	..	..
VI	..	..	..	..	390	0.3	..	..
Total	1408	0.4	1374	1.2	2598	1.0	505	4.0

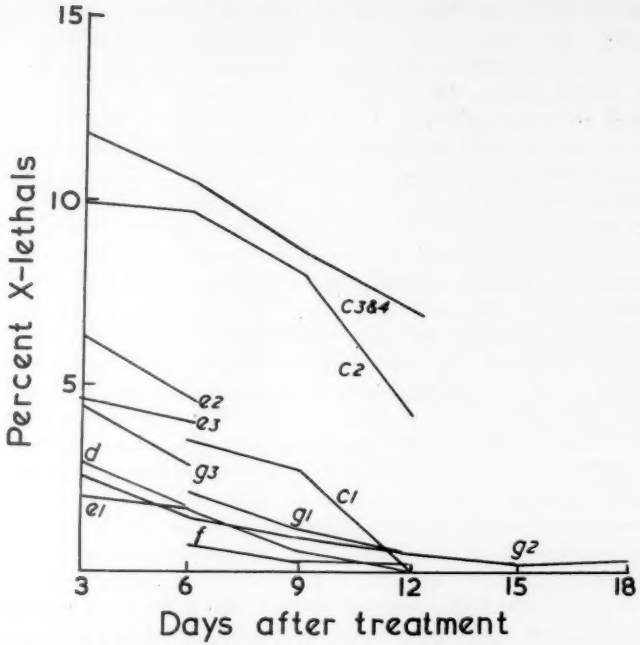

FIGURE 2.—The brood-mutation curves for the dimesylates. Curves lettered to correspond with experiments in Table 2.

of doses to be analysed. Furthermore, even with the tolerated doses used, sterility through cytotoxicity (cell killing) often occurred and was more drastic the later was the brood. In some experiments, therefore, it was only possible to test two broods, though in most instances the experiments could be extended to four fractions.

The trend of the variation in the mutation rate with brood for the dimesylates is fundamentally the same as that for the monomesylates. The mutation rate decreased steadily the later was the brood. In one experiment (CB.2095, g2 Table 2) it was possible to recover and test a reasonable chromosome number for six broods. The mutation rate in the last two broods, however, was not significantly above control.

The x distribution for the brood-mutation fluctuations under the dimesylates is given in Table 4B. The gradual fall in the mutation rate from the first to the fourth brood is most consistent between experiments with the same, as well as with different compounds. This is portrayed in the higher significance of the interbrood x in the combination test with this subseries than with the monomesylates. On the other hand, the gross depression from the fourth to the fifth brood which is characteristic of the monomesylates is not revealed by the x-combi-

nation test for the dimesylates. This, however, is fortuitous and is due to the fact that the experiment which permitted the recovery of sperm for the later progeny was with a weakly mutagenic compound (CB.2095, g2 Table 2 and Figure 2) which gave in the fourth brood a mutation rate slightly above the control level. It is clear, therefore, that any further drop in this rate in the later broods could only be slight and insignificant, even if the germ cells providing the later progeny were mutagenically refractory.

Dimethanesulphonylalkanols: This is a new series of dimesyloxy esters in which the central part of the molecule is a 'sugar' moiety. These compounds proved to be almost completely nontoxic to the treated flies, and their sterilizing effect (through cytotoxicity) was also negligible at moderate doses. These technical advantages enabled the assessment of the cell stage effect under these derivatives for the whole germ line, up to ten broods (Table 3).

An inspection of the brood-mutation curves (Figure 3) for the different experiments with the polyol-mesylates, reveals a greater variability than with the mono- and dimesylates. Nevertheless, it is clear that experiments conducted at moderate mutagenic levels (curves j and h2, Figure 3) give virtually identical brood-mutation curves. These curves differ from that for the highest mutagenicity experiment (h3, Figure 3) and to a lesser extent from those with the lowest mutation rates (h1 and i, Figure 3). This suggests that one of the factors confusing the brood-mutation relationship may be a function of the strength of the treatment, which, in turn, depends on the level of mutagenic activity and the dose. A consideration of the experiments with the most active member of the series (the mannitol derivative: CB.2511) shows that the most anomalous brood-mutation pattern did, indeed, occur at the highest dose. It was further noticed that at this dose extreme sterility occurred in the fourth and fifth broods, thus permitting the recovery of only a few chromosomes for the genetic test (h3,

TABLE 3

Sex-linked recessive lethals induced in the successive broods (three-day intervals) by various polyol-mesylates

	CB.2511						CB.2562		CB.2628	
	h1		h2		h3		i		j	
	1×10^{-2}M		8×10^{-2}M		16×10^{-2}M		16×10^{-2}M		16×10^{-2}M	
Brood	chr.	%1	chr.	%1	chr.	%1	chr.	%1	chr.	%1
I	548	2.0	531	5.5	130	11.5	526	1.0	481	6.7
II	521	2.1	533	3.8	323	13.9	516	1.9	490	3.5
III	544	0.7	531	4.9	280	16.8	531	0.9	428	4.2
IV	524	0.6	531	8.3	92	17.4	533	2.1	467	7.3
V	548	0.7	508	2.2	46	17.4	519	1.3	487	4.1
VI	543	0.4	1066	1.9	430	0.0	518	1.0	475	2.1
VII	549	0.2	..	..	524	0.0	524	0.0	477	1.9
VIII	..	..	..	..	..	..	..	..	523	0.2
IX	..	..	..	..	..	..	..	..	499	0.2
X	..	..	..	..	..	..	..	..	495	0.8
Total	3777	1.0	3700	4.1	1825	7.2	3667	1.2	4822	3.0

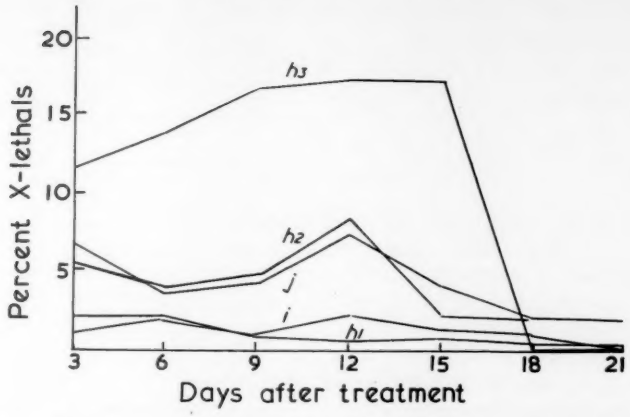

FIGURE 3.—The brood-mutation curves for the polyol-mesylates. Curves lettered to correspond with experiments in Table 3.

Table 3). This indicates that the strong treatment has induced differential cytotoxicity, resulting in the elimination of some stages of the male germ line. This is bound to result in disturbances in the sampling of the cell stages during progeny fractionation, which would lead to an anomalous brood-mutation relationship.

In view of the variation in the brood-mutation pattern between experiments under the polyol-mesylates, it is only possible to assess their average trend of behaviour and determine how far it differs from the other chemical subseries. This can best be inferred from the statistical analysis of the degree and direction of the brood fluctuations in mutation rate detailed in Table 4C. The average brood-mutation trend as revealed by the x-combination test is that the mutation rate falls gradually (though insignificantly) from the first to the third brood, rises significantly from the third to the fourth and then falls in later broods till it reaches the control level. The combination test was also undertaken with the exclusion of the anomalous experiment with the highest mutagenicity (h3, Table 4C), but the data still revealed essentially the same brood-mutation relationship.

A feature of difference in the above pattern as compared to that for the mono- and dimesylates, is that the mutation rate appears to remain the same for the first three broods, instead of falling consistently. There are indications, however, that this difference may not be real; in one experiment (j, Table 4C) the drop from the first to the second brood is decisively significant ($P < 0.01$) and in another (h1, Table 4C) the decrease from the second to the third brood is also significant ($P < 0.03$). It is feasible, therefore, that further experimentation may permit the exclusion of this apparent feature of difference in the brood pattern of the polyol-mesylates.

The outstanding deviation of the mutation pattern of the polyol derivatives, as compared to the other sulphonate subseries, is that the level of mutagenic activity

TABLE 4

The distribution of χ for interbrood fluctuations of the mutation rate in the separate experiments with individual mesyloxy esters and the combination tests for the various chemical subseries

Subseries	Compound	Exp.	Broods I-II	II-III	III-IV	IV-V	V-VI	VI-VII
A: monomesylates	CB.1528	a1	−1.42	0.37	−1.36	−6.14	0.50	0.51
		a2	−1.02	−0.83	1.70	−6.83	0.23	−1.76
		a3	−0.94	−0.47	−1.41	−2.54	...	...
	CB.1540	b1	−0.38	−0.38	−0.91	−1.66	−2.65	−0.48
		b2	−0.58	−1.02	0.22	−4.50	−1.71	−0.87
	$\Sigma\chi/\sqrt{n}$		−1.94	−1.04	−0.79	−9.67	−1.82	−1.30
	P		0.03	0.15	0.20	$<10^{-9}$	0.04	0.10
B: dimesylates	CB.2040	c1	...	−0.73	−0.68	...	...	...
		c2	−0.08	−0.76	−1.62	...	...	...
		c3	−0.50	−0.70	−0.46	...	...	...
		c4	...	−0.76	−0.93	...	...	...
	CB.2041	d	−0.73	...	...	...	...	...
	CB.2058	e1	−0.44	−1.71	−1.38	...	...	...
		e2	−0.87	...	...	...	...	...
		e3	−0.51	...	...	...	...	...
	CB.2094	f	...	−0.93	0.11	...	...	...
	CB.2095	g1	...	−1.23	−0.99	...	...	...
		g2	−1.26	−0.65	−0.75	−0.60	0.07	...
		g3	−0.96	...	...	...	...	...
	$\Sigma\chi/\sqrt{n}$		−1.89	−2.64	−2.37	−0.60	0.07	...
	P		0.03	<0.01	<0.01	0.27	0.47	...
C: polyol-mesylates	CB.2511	h1	0.56	−1.91	−0.03	0.03	−0.84	−0.59
		h2	−1.33	0.92	2.23	−4.40	−0.04	...
		h3	0.68	0.97	0.13	0.00	−7.78	0.00
	CB.2562	i	1.34	−1.36	1.50	−0.89	−0.58	−2.27
	CB.2628	j	−2.27	0.58	1.90	−2.12	−1.79	−0.24
	$\Sigma\chi/\sqrt{n}$		−0.46	−0.36	2.56	−3.29	−4.92	−1.55
	P		0.33	0.36	<0.01	$<10^{-3}$	$<10^{-6}$	0.06

in the fourth brood is high, usually above that in the third (Table 4C, III-IV), being roughly of the same order as that occurring in the first. To determine the exact statistical significance of the above feature, a direct comparison of the mutation rate in the first relative to the fourth broods was undertaken for the separate experiments in a series of 2×2 tables. There was no evidence for a significantly higher mutation rate in the fourth brood, either in the separate tables or after combination ($\Sigma\chi = 2.86 \pm 2.24$; $P = 0.1$).

The early germ cells which are responsive to the polyol-mesylates are apparently not exclusively sampled in the fourth brood. The slight rise from broods two to three in experiments h2 and j (Figure 3, Table 4C) may well be due to the earlier recovery of these cells. Conversely, the high mutation rate in broods

as late as the fifth (Table 3) is most likely to be due to the delayed utilization of some responsive cells in admixture with the earlier refractory stages. In the highest dose experiment with the mannitol derivative (h3, Table 3), the utilization of the refractory cells was delayed entirely until the sixth brood, while the responsive cells were used in the fourth and fifth broods.

DISCUSSION

The analysis of the mutagenic properties of the most diverse alkylating mutagens has now given a rough idea about the speed of utilization of the various stages of the male germ line in our standard brood-fractionation technique. Sperm derived from postspermatogonial stages is mainly used during the first four broods (0–12 days after treatment) while that from the spermatogonial stages is utilized in the later progeny (FAHMY and FAHMY 1954, 1955, 1956b, 1960b,c). Evidence that this sampling also applies to the mesyloxy esters is apparent from an inspection of the brood-mutation curves especially under the most active members of the series: the monomesylates. A very sharp and consistent interbrood gradient occurs between the fourth and fifth broods, bringing the mutation rate down almost to the control level in the late progeny. This indicates a major transition from responsive to refractory cells. Since the refractory cells continue to be sampled till the end of fractionation, they must represent the earliest stages of the germ line, namely the spermatogonia. The responsive cells used in the preceding four broods must, accordingly, be postspermatogonial stages; namely, spermatocytes undergoing meiosis, and spermatids in spermateleosis.

The pattern of cell-stage response under the polyol-mesylates, though on the whole in conformity with that for other mesyl-esters, is far less consistent and is subject to variation according to the strength of the treatment. In harmony with other mesyl-esters, the mutation rate is high for the postspermatogonial stages and low for the gonia. A unique feature of the polyol derivatives, however, is that they exert high activity also on the earliest postspermatogonial stages (the fourth brood), which are presumably spermatocytes. It is, perhaps, significant that the high response of these cells is also manifested in cytotoxicity; at the highest dose of the mannitol derivative, extreme sterility (through germ-cell killing) coincided with high mutagenicity. It would seem, therefore, that the relatively high activity of the polyol-mesylates on the cells of the fourth brood is a general phenomenon which is by no means restricted to mutagenesis. The reason for this is unknown, but it could simply be a function of cell permeability, resulting in a higher concentration of the drug in the fourth brood cells; thus resulting in a nonspecific general rise in biological activity. It should be noted, however, that the mutation rate in the fourth brood does not rise significantly above that in the first. Whatever the reason is for the high response in the fourth brood, it clearly has not obliterated the important feature that mature sperm is one of the most responsive stages under the polyol derivatives, as it is under the other compounds studied of the mesyloxy series.

A general consideration of the present data reveals that there is an overriding trend of cell-stage response which may be typical of the mesyloxy series. Mutagenicity is high among the meiotic and postmeiotic stages with maximal response in the mature sperm, while the spermatogonia are virtually refractory. It is of interest that this pattern was also manifest even when the compounds were administered orally (with the food) to adult males (RÖHRBORN 1959a,b). The compounds tested in this way were 'Myleran' (CB.2041) and its dimethyl derivative (CB.2348), as well as the monomesyl ester of butanediol. The inadequate method of administration and the low activity of the compounds tested did not permit the achievement of high mutation rates in RÖHRBORN experiments. Nevertheless, with the feeding technique, as in our injection experiments, there was clear evidence of higher activity in the earlier than in the later progeny fractions.

A fundamental issue that arises in relation to the analysis of the mutagenic cell-stage response under chemical compounds by progeny fractionation is whether the time factor plays a role in raising the mutation rate in the later broods. With highly reactive compounds (like the mustards) there can be little doubt that mutagenic action is ephemeral and simultaneous on the various cell stages of the testis; these compounds are completely inactivated (by hydrolysis) in the tissues of the treated flies long before the first sperm sample is drawn for the genetic tests (FAHMY and FAHMY 1960c). With highly stable compounds like the mesyloxy esters, however, the retention of the active compound in the tissues for many days may occur, and this could indeed favour a higher mutation rate in sperm used in later matings due to longer time of treatment. For the mono- and dimesylates, the above complication did not conceal the fact that mutagenic cell-stage response is lower the younger is the cell stage. It should nevertheless be borne in mind, that the actual difference in cell sensitivity (at least for the stages sampled in some of the earlier broods) may well be more pronounced than the interbrood mutation gradients indicate. With the polyolmesylates on the other hand, it may be argued that the level or rising mutation curves (Figure 3 and Table 4C) for the first four broods may be due to longer time of treatment, as a result of intracellular retention of the mutagenic compound. However, it is known that the hydrolysis rate *in vitro* of the polyolmesylates is roughly twice as fast as that for some of the dimesylates (such as CB.2040 and 2041); this would hasten their destruction in the tissues and thus lessen the role of compound retention. Since this factor did not conceal the pattern of cell-stage response under the more stable dimesylates, there is no reason to suspect that it does so with the polyol derivatives.

The cell-stage effect under the mesyloxy esters is substantially different from that of other chemical series of the alkylating mutagens. The epoxides, imines and carboxylic-acid mustards, while resembling the mesyl esters in being active only on the postmeiotic stages, do nevertheless present a feature of major difference. These agents give a peak of maximal activity on a postmeiotic stage (probably an early spermatid) where the mutation rate reaches a level significantly above that induced in the sperm (BIRD and FAHMY 1953; FAHMY and FAHMY 1955, 1958a,b, 1960b). The mustard derivatives of amino acids (particularly

those with 'natural' molecular configuration) give a pattern of cell-stage response which is the reverse of that produced by the mesyloxy esters. With these 'mustards' high activity occurs in the spermatogonia, and minimal effect is detectable in mature sperm (FAHMY and FAHMY 1960b,c).

An outstanding deviation from the pattern of mutagenicity manifested by the mesyloxy esters occurs with the halogeno-alkyl derivatives, particularly 2-chloroethyl methanesulphonate. Although these derivatives, like other members of the series, owe their alkylating potentiality to the mesyloxy group, yet they show the reverse pattern of cell-stage response—maximal effect is given in the spermatogonia instead of the sperm (FAHMY and FAHMY 1956, 1957). Chemical and genetic evidence were available, however, indicating that the atypical mutagenicity pattern of the chloroester was due to its conversion *in vivo* to an amino acid mustard, probably 2-chloroethylcysteine. The latter mustard did, indeed, prove to be mutagenically specific to the spermatogonia, thus supporting the conversion hypothesis (FAHMY and FAHMY 1960c).

In contrast to the above situation with the halogeno-alkyl sulphonates, many changes in the chemical structure of the prosthetic moiety of the mesyloxy esters did not result in major alteration of the pattern of cell-stage response. The chemical changes in the nonalkylating moiety here investigated included chain length, valency unsaturation, steric configuration and hydroxyl substitution. While slight differences in the pattern of cell-stage mutagenicity did accompany the above chemical changes; yet, an overriding general trend was discernible. The slight differences in detail between chemical subseries may conceivably be attributed to physical factors, mainly differences in the permeability of the cell membranes of the stages of the germ line to the various compounds. The characteristic over-all pattern of cell-stage effect, on the other hand, differentiates the mutagenic mode of action of the mesyloxy esters analysed from that of other chemical series of the alkylating mutagens. This pattern, therefore, is likely to be a genuine manifestation of a characteristic biochemical mechanism of interaction between compounds of the mesyloxy series and the genetic material during sperm differentiation.

SUMMARY

The analysis of the fluctuation of the mutation rate in the fractionated progeny of males treated with various mesyloxy esters revealed a common trend in the pattern of cell-stage response. The postspermatogonial stages are responsive, with maximal effect on the mature sperm, while the spermatogonia are refractory. Variation did occur, however, in the relative response of the postspermatogonial stages (spermatids and spermatocytes) to different chemical subseries. With the mono- and dimesylates these stages show a decreased response the earlier is the stage of germ-cell differentiation. The polyol-mesylates exert on an early postspermatogonial stage (presumably spermatocytic) a mutagenicity equivalent to that in mature sperm.

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PREFERENTIAL SEGREGATIONS IN TRIPLOID FEMALES[1,2]

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RECENT work has shown that regular preferential segregations involving the following chromosome pairs can occur in diploid females: Y-II (OKSALA 1958), Y-IV (GRELL 1957, 1959b), X-II (FORBES 1960), Dup(X)-IV (GRELL and GRELL 1960), and X-IV (GRELL 1959a). In all these experiments specific chromosomes were made to behave as univalents with respect to their homologues by the use of translocations and inversions (see OKSALA 1958, for an interesting interpretation of these preferential segregations based on the formation of a polarized bouquet stage in prophase). The particular advantage of triploid females is that the same type of preferential segregations can be observed without the necessity of employing gross chromosomal rearrangements. New information is presented here on chromosome segregation in triploid females which provides additional evidence for interactions between non-homologous chromosomes.

Triploid females provided the first indication that the four different chromosomes in *Drosophila melanogaster* did not always segregate independently of each other. In triploids with three free X chromosomes (referred to in the future as free X triploids) those recovered eggs with two X chromosomes almost always had one set of autosomes, and the recovered eggs with one X chromosome almost always had two sets of autosomes (BRIDGES, in BRIDGES and ANDERSON 1925). Further work with respect to preferential segregations in triploids has been limited to the observations of BEADLE (1934, 1935) on triploid females with two attached-X chromosomes and one free X chromosome (referred to in the future as attached-X triploids). These females showed the same type of preferential segregation observed in free X triploid females, but it was much less marked.

BEADLE discovered the occurrence of X chromosome nondisjunction in attached-X triploid females (1935) and pointed out that here also the distribution of the X chromosomes was not random with respect to the autosomes. The nullo X eggs almost always had two sets of autosomes (resulting in X3A patroclinous metamales when fertilized by X1A sperm) and rarely had one set of autosomes (resulting in X2A patroclinous diploid males when fertilized by X1A sperm). On a random basis, the two types of eggs should have been equally frequent.

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In earlier work (1934) many of the attached-X triploid females studied by BEADLE carried a Y chromosome. As a result of secondary nondisjunction, patroclinous diploid males were very frequent, and this was one of the criteria used by BEADLE to separate the Y-bearing from the non-Y-bearing triploid female parents. Neither regular metamales (resulting from X2A eggs and Y1A sperm) nor patroclinous metamales were found by BEADLE (viability of metamales is low and variable), so the relative frequency of 01A eggs *versus* 02A eggs could not be determined. When regular disjunction of the X chromosomes occurred the segregation of the X chromosomes appeared to be nearly random with respect to the autosomes.

Attached-X triploid females were also studied in the present experiments. The use of a marked Y chromosome permitted a study of the segregation of the Y chromosome itself, as well as a more complete analysis of the influence of the Y chromosome on X chromosome segregation. The influence of a free X chromosome carrying multiple inversions on the frequency of primary nondisjunction was also studied. In addition some new information is presented on the types and frequencies of autosomally unbalanced eggs formed by attached-X triploid females.

METHODS

In the basic triploid stock used in these experiments the triploid females carried attached-X chromosomes and a free X chromosome. The attached-X chromosomes were homozygous for the genes yellow^{2} (body color), scute (removes postscutellar bristles), apricot (eye color), and echinus (large rough eyes), and the free X chromosome carried the markers yellow^{31d}, scute^{8}, diminutive (small body and bristle size), and Bar (shape of eye). The free X chromosome was the FM4 (First Multiple 4) chromosome of MISLOVE (MISLOVE and LEWIS 1954). This chromosome carried an X-ray induced inversion superimposed on two other inversions already present, *In(1)sc^{s}*, and *In(1)*dl-49. The females used to start the stock had been tested to be sure they did not carry a Y chromosome (certain exceptions are noted below). The males in the basic stock carried the FM4 chromosome and a Y chromosome carrying a short duplication of the tip of the X chromosome with the wild type allele of yellow (the *sc^{s}*·Y).

To obtain triploid females with normal free X chromosomes, the stock females were mated to males with normal X chromosomes carrying yellow and white (eye color). To obtain triploid females with a Y chromosome, the stock females were mated to males carrying the appropriate X chromosome (FM4, or *y w*) and both the normal Y and the *sc^{s}*·Y. Triploid offspring carrying the *sc^{s}*·Y could be picked out because of their "not yellow" phenotype.

In experimental matings the males carried an X chromosome with yellow. If the triploid female carried a *sc^{s}*·Y chromosome the males carried a normal Y chromosome, if the triploid females did not have a Y chromosome, the males carried a *sc^{s}*·Y chromosome. The scheme for identifying chromosomal origins among the offspring went thus: yellow *versus* wild type determined the absence

or presence of a sc^8·Y chromosome, white (or apricot or Bar) *versus* wild type determined maternal *versus* paternal origins of the X chromosomes. All crosses were made at 25 ± 1°C and offspring were counted from eggs laid during two consecutive five-day periods except for the experiments shown in Table 2, no. 1 and no. 6, where 3–5 such transfers were made. The latter two experiments also furnished data for Table 1.

TABLE 1

Effect of age on segregation patterns shown by triploid females

Type of triploid parent and age in days		Total 2N females produced	Percent attached-X 2N females	Percent exceptional 2N males	Percent exceptional metamales
$\hat{XX}$,X					
	1–5	2883	56.1	0.1	0.0
	6–10	2556	54.3	0.4	0.1
	11–15*	432	54.9	0.0	0.5
Total	1–10	5439	55.2	0.2	0.0
$\hat{XX}$,FM4					
	1–5	4035	60.1	1.0	..†
	6–10	3789	59.1	1.0	..†
Total	1–10	7824	59.6	1.0	..†
$\hat{XX}$,FM4,sc^8·Y					
	1–5	367	48.5	10.1	3.3
	6–10	277	52.0	13.7	2.9
	11–15	234	48.3	10.7	5.1
Total	1–15	878	49.5	11.4	3.6

* Several experiments involving $\hat{XX}$,X triploids were pooled, but only one provided data for the 11–15-day age range.
† This class was not counted.

Methods used for the crosses from which the data for Table 6 was obtained have been reported by FROST (1961). All other information recorded in this paper was obtained from crosses using single triploid females per one ounce creamer. The results shown for no. 1 of Table 2 were obtained in crosses where the possibility of the triploid female parents carrying a Y chromosome had not yet been excluded. However such females could usually be detected because of the high rate of secondary nondisjunction. One female producing five exceptions was excluded and another female producing three exceptions was also excluded as the exceptional males were fertile. Of the remaining 303 females, 32 produced the total of 36 exceptions recorded in Table 2, four triploid females producing two exceptions. Exceptional diploid males from seven different triploid females were tested, the males from only the one female mentioned above being fertile.

Analysis of autosomally balanced eggs

Effect of age: There are four different types of segregation that produce autosomally balanced eggs, two which result in regular eggs, X1A-$\hat{XX}$2A and

TABLE 2

Autosomally balanced egg types produced by different types of triploids

	X Chromosome constitution of eggs and recovered zygotes												
	Regular X chromosome segregations								Nondisj. X chromosome segregations				
		X1A — $\hat{X}$X2A			$\hat{X}$X1A — X2A				01A — $\hat{X}$XX2A			X$\hat{X}$X1A — 02A	
Triploid type	XX2A	X2A	X$\hat{X}$X3A	$\hat{X}$X3A	X$\hat{X}$X2A	$\hat{X}$X2A	XX3A	X3A	X2A	X$\hat{X}$XX3A	X$\hat{X}$X3A	X$\hat{X}$X2A	X3A	Total
1. $\hat{X}$X,X	4365	3956	2842	1683	8	5389	3722	33	15	1	8	..	12	22034
Percent	19.8	18.0	12.9	7.6	0.0	24.5	16.9	0.1	0.1	0.0	0.0	..	0.1	
2. $\hat{X}$X,FM4	133	113	84	59	..	191	139	5	2	1	..	..	19	746
Percent	17.8	15.1	11.3	7.9	..	25.6	18.6	0.7	0.3	0.1	..	..	2.5	
3. $\hat{X}$X,FM4*	188	222	..	..	..	320	..	..	5	..	..	..	47	782
4. $\hat{X}$X,X	151	163	173	85	..	122	162	16	23	5	10	..	9	919
sc^{8}-Y Percent	16.4	17.7	18.8	9.2	..	13.3	17.6	1.7	2.5	0.5	1.1	..	1.0	
5. $\hat{X}$X,X†	267	194	210	119	2	174	201	12	10	2	7	2	..	1200
sc^{8}-Y Percent	22.3	16.2	17.5	9.9	0.2	14.5	16.8	1.0	0.8	0.2	0.6	0.2	..	
6. $\hat{X}$X,FM4	208	131	172	77	..	111	177	1	40	8	23	..	15	963
sc^{8}-Y Percent	21.6	13.6	17.9	8.0	..	11.5	18.4	0.1	4.2	0.8	2.4	..	1.6	

* In this experiment only diploid offspring and exceptional metamales were counted.
† In this experiment the triploid females were mated to FM4,Y males instead of to *y*,Y males.

$\hat{X}\hat{X}$1A-X2A, and two which result in nondisjunctional eggs, 01A-$\hat{X}\hat{X}$X2A and $\hat{X}\hat{X}$X1A-02A. In Table 1 the following four types of offspring are used as a measure of the relative frequency of the four types of segregation: free X diploid females (X1A egg, X1A sperm) for the X1A-$\hat{X}\hat{X}$2A segregation, attached-X diploid females ($\hat{X}\hat{X}$1A egg, Y1A sperm) for the $\hat{X}\hat{X}$1A-X2A segregation, exceptional diploid males (01A egg, X1A sperm) for the 01A-$\hat{X}\hat{X}$X2A segregation, and exceptional metamales (02A egg, X1A sperm) for the $\hat{X}\hat{X}$X1A-02A segregation. Metamales are the most viable type of offspring produced by this last segregation. 02A eggs fertilized by Y-bearing sperm form lethal zygotes, as do $\hat{X}\hat{X}$X1A eggs fertilized by X-bearing sperm. $\hat{X}\hat{X}$X1A eggs fertilized by Y-bearing sperm form diploid metafemale zygotes which are much less viable than metamale zygotes. The total number of free X and attached-X diploid females produced is used as a base line with which to compare the relative frequencies of offspring coming from the regular and nondisjunctional segregations. For any one experiment in Table 1, only those triploid females producing offspring in all age categories are compared.

Table 1 shows no effect of age on the pattern of segregations occurring in triploid females, at least for the 1–15 day period studied. REDFIELD (1930) also found that crossing over frequencies in triploids were not affected by age.

Nondisjunction of the X chromosomes: Practically all of the autosomally balanced eggs recovered from attached-X triploid females with a normal free X chromosome come from the two regular segregations. BEADLE found that only 0.2–0.3 percent of the total offspring came from the two nondisjunctional segregations, a result that agrees well with the data presented here in Table 2 no. 1. The proportion of exceptional offspring can be raised markedly, however, by introducing a Y chromosome (Table 2 no. 4 and no. 5), as BEADLE showed with respect to the production of exceptional diploid males.

What Table 2 shows in addition to previously known results is that the frequency of nondisjunction can also be raised by introducing a free X chromosome with multiple inversions and that the pattern of nondisjunction shown in this case is distinctly different from that shown when a Y chromosome is present. If attached-X triploid females with a normal free X chromosome (no. 1) are compared to those with the FM4 chromosome (no. 2) it can be seen that the frequency of exceptional diploid males is increased very slightly from 0.1 percent to 0.3 percent, while the frequency of exceptional metamales is increased from 0.1 percent to 2.5 percent. Rounding off the percent figures to the nearest tenth obscures some of the difference. Comparing the number of exceptions to the total number of regular diploid offspring, there is a 50–70-fold increase in frequency of exceptional metamales, but only a 4–6-fold increase in frequency of exceptional diploid males. There is almost a tenfold difference in frequency of exceptional diploid males *versus* exceptional metamales in the offspring of triploids carrying the FM4 chromosome (no. 2). Thus almost the entire increase in nondisjunction frequency is attributable to the $\hat{X}\hat{X}$X1A-02A segregation rather than the 01A-$\hat{X}\hat{X}$X2A segregation. On the other hand, in triploid females carrying a

Y chromosome (no. 4, no. 5, and no. 6) much of the increase in frequency of nondisjunction is also due to the 01A-XXX2A segregation.

Estimated frequencies of the four types of segregations: Because of differences in viability, it is difficult to properly assess the relative frequency of the nondisjunctional segregations by simply examining the numbers of offspring recorded in Table 2. For this reason estimated frequencies of both the regular and nondisjunctional types of segregation are given in Table 3. The methods used in making these estimations are discussed below in detail since they also illustrate why, in certain cases, conclusions regarding the relative frequency of the two regular segregations are not justified.

One assumption used in making the estimations for Table 3 was that the two types of eggs resulting from any one segregation were equally frequent. Whether this is actually true cannot be determined. For example, in the columns under the X1A-$\hat{X}\hat{X}$2A segregation in Table 2, triploid females in no. 1 and no. 2 occur less frequently than the diploid males or females. This difference could be interpreted in terms of lower viability of triploid females or in terms of a preferential orientation of the first meiotic division so that the $\hat{X}\hat{X}$2A nucleus was preferentially included in the polar body. Estimations based on the latter interpretation would change the frequencies shown in Table 3 very little, at least for those triploid females without the Y chromosome. Even marked preferential orientations occurring in the nondisjunctional segregations would not affect their estimated frequency greatly. If, because of a preferential orientation, 01A eggs were twice as frequent as $\hat{X}\hat{X}$X2A eggs, an estimate of the total number of eggs based on the recovery of 01A eggs only, but which assumed equality of the two types of eggs, would be one and one-third times too large.

Other assumptions used in making the estimates for Table 3 were that X and Y sperm fertilized eggs in a 1:1 ratio, and that diploid offspring (with one exception) were 100 percent viable. Thus for the X1A-$\hat{X}\hat{X}$2A segregation the diploid

TABLE 3

Estimated frequencies in percent of the four segregation types resulting in autosomally balanced eggs

Type of triploid female	Viability of metamales	Regular X chromosome segregations		Nondisjunctional X chromosome segregations	
		X1A-$\hat{X}\hat{X}$2A	$\hat{X}\hat{X}$1A-X2A	01A-$\hat{X}\hat{X}$X2A	$\hat{X}\hat{X}$X1A-02A
$\hat{X}\hat{X}$,X	.006	36	47	0	17
	.130	43	56	0	1
$\hat{X}\hat{X}$,FM4	.026	12	18	0	70
	.130	27	41	0	32
$\hat{X}\hat{X}$,X,*sc*^{8}·Y*	.068	72		4	24
	.130	81		5	14
$\hat{X}\hat{X}$,FM4,*sc*^{8}·Y	?				
	.130	73		7	20

* These estimates are based on the results shown in Table 2, No. 4.

males and females were added together and multiplied by two, for the $\widehat{XX}$1A-X2A segregation the number of attached-X diploid females was multiplied by four, and for the 01A-$\widehat{XX}$2A segregation the number of exceptional diploid males was multiplied by four.

A correction was made however, in the number of attached-X diploid females produced by triploid females carrying a Y chromosome. In Table 2 in the columns under the $\widehat{XX}$1A-X2A segregation, free X intersexes occur less frequently than attached-X diploid females among offspring of triploid females without a Y chromosome. This would be expected since intersexes have lowered viability, but for the triploid females with a Y chromosome the situation is reversed. The most probable explanation seems to be that the viability of attached-X diploid female offspring receiving the maternal Y chromosome, as well as the paternal Y chromosome, is greatly lowered, and most attached-X diploid females do receive the maternal Y chromosome (see the next section). NOVITSKI (1954) discovered a similar effect when studying a tandem acrocentric attached-X chromosome. The latter chromosome had most of the Y chromosome attached to it and NOVITSKI found that twice as many attached-X female offspring were recovered when these females were mated to X·Y/0 males rather than to X/Y males, an effect which he ascribed to the lowered viability of attached-X females hyperploid for the heterochromatin of the X and Y. COOPER (personal communication) has studied $\widehat{XX}$–*sc*^{8}·Y nondisjunction in attached-X females and finds that exceptional female offspring are recovered 3–5 times more frequently when the females are mated with X·Y/0 males rather than with X/Y males, although other experiments of his indicate that it is not simply hyperploidy for the Y chromosome that is responsible for these altered frequencies. The above findings suggest that if the triploids with the Y chromosome had been mated to X·Y/0 males, attached-X diploid female offspring would have been recovered with a much higher frequency, and that in these triploid crosses the viability of attached-X females receiving both Y chromosomes is low. An alternate explanation based on a preferential orientation of the first meiotic division is discussed in the next section, but such an explanation would not alter the conclusions reached at the end of this section since the estimated number of $\widehat{XX}$1A-X2A segregations would be lowered and the relative frequency of the nondisjunctional segregations would be raised. Since most intersexes do not receive the maternal Y chromosome it was assumed that the viability of free X intersexes was unaffected by the presence of a Y chromosome in the triploid female parent. The expected number of attached-X female offspring could then be derived from the relative frequency of attached-X females and free X intersexes observed among the offspring of triploid females without a Y chromosome.

Table 2 shows another possible influence of the Y chromosome that would lead to errors in estimating the number of X1A-$\widehat{XX}$2A segregations, but for which no corrections were made. From triploid parents without the Y chromosome (no. 1 and no. 2), triploid offspring are recovered with a somewhat lower frequency than diploid males, but from triploid parents with the Y chromosome

(no. 4, no. 5, and no. 6) triploid offspring are somewhat more frequent than diploid males. Estimates of the frequency of the X1A-$\hat{X}\hat{X}$2A segregation would vary according to one's interpretation of these differences, i.e. as changes in the relative viability of the diploid males, or as an influence of the Y chromosome on the preferential orientation of the first meiotic division. The possible errors here would not greatly affect the relative magnitude of the nondisjunctional segregations *versus* the regular segregations, but they would affect estimates of the relative magnitude of the two regular segregations. For this reason the estimates of the two regular segregations made for Table 3 are not shown separately.

BEADLE (1934) had observed that in triploid females without a Y chromosome attached-X diploid female offspring were 1.6 times as frequent as free X diploid females, while in triploid females carrying a Y chromosome, attached-X diploid female offspring were slightly less frequent than free X diploid females. This led to his observation that the segregation of the X chromosomes appeared to be nearly random with respect to the autosomes in triploid females carrying a Y chromosome. The present experiments show that it is not yet possible to say whether the two different regular segregations in these females are equally frequent or not.

The only type of offspring consistently recovered from the $\hat{X}\hat{X}$X1A-02A segregation is the exceptional metamale (X1A sperm and 02A egg) and to estimate the frequency of this segregation one must estimate the viability of metamales. Metamales also result from X2A eggs and the number of these regular metamales can be compared to the number of attached-X diploid females since they both come from the same type of segregation. In the case of those triploid females carrying a Y chromosome the number of regular metamales was compared to the corrected number of attached-X diploid females. Viabilities calculated in this way are shown in Table 3 (the first figure given for each type of triploid female) and were the basis for determining the number of $\hat{X}\hat{X}$X1A-02A segregations in each experiment. These viabilities may be compared with values of 0.13 and 0.09 calculated from the data of BRIDGES (BRIDGES and ANDERSON 1925) and BEADLE (1935) respectively, and to give an idea of the possible range of error the value of 0.130 is taken as a maximum estimate of metamale viability. Consequently, Table 3 also shows the estimated frequency of $\hat{X}\hat{X}$X1A-02A segregations calculated on the latter basis. Whether viability of regular metamales represent good estimates of viability of exceptional metamales is particularly in doubt for those triploid females carrying the FM4 chromosome. Exceptional metamales are wild type or yellow only, while regular metamales are yellow^{31d} (or wild type), scute^{8}, diminutive, and Bar. Some evidence for the lower viability of FM4 metamales is provided by Table 2 no. 5 where the triploid female parents had been mated to FM4 males instead of to y males. Consequently the exceptional metamales would carry the phenotype associated with the FM4 chromosome. No exceptional metamales were recovered but the recovery of two exceptional diploid metafemales suggests that the $\hat{X}\hat{X}$X1A-02A segregation had been relatively frequent.

Despite the large errors Table 3 makes clear the following points:

1. There is an appreciable frequency of $\hat{X}\hat{X}$X1A-02A segregations even when no Y chromosome is present and the free X chromosome is structurally normal. BEADLE's 1935 data yield an estimate of four percent for the frequency of the $\hat{X}\hat{X}$X1A-02A segregation and thus permit the same conclusion.

2. When the FM4 chromosome is substituted for the structurally normal free X chromosome more than 30 percent of the segregations leading to autosomally balanced eggs are due to the $\hat{X}\hat{X}$X1A-02A type.

3. In the absence of a Y chromosome the frequency of the 01A-$\hat{X}\hat{X}$X2A segregation is negligible, and while the introduction of a Y chromosome causes substantial increases in the frequency of the 01A-$\hat{X}\hat{X}$X2A segregation, $\hat{X}\hat{X}$X1A-02A segregations are 3–6 times more frequent.

Preferential segregation of the Y chromosome: Table 4 shows the distribution of the Y chromosome among the progeny of the Y carrying triploid females listed in Table 2 no. 4, no. 5, and no. 6, the results for no. 4 and no. 5 being pooled together. The Y chromosome is recovered about 90 percent of the time from eggs with one set of autosomes, a distribution suggesting that when a sex chromosome had not paired with a homologue it had usually paired with an autosome. The only exception to the rule that the Y chromosome is recovered preferentially at the 1A pole is the $\hat{X}\hat{X}$X1A-02A segregation where the Y chromosome is almost always recovered at the 02A pole. The behavior of the Y chromosome in this case is that predicted from its role in producing nondisjunction. The reasons for coming to the above conclusions are discussed in detail later. Table 4 gives no indication that the Y chromosome pairs preferentially with one of the X chromosomes. If it did the distribution of the Y chromosome with respect to the autosomes should have been markedly different in the two types of regular X chromosome segregations.

Table 4 provides more information regarding the effect of the presence of the Y chromosome on the number of attached-X diploid female offspring produced by triploid females. For every $\hat{X}\hat{X}$1A egg without a Y chromosome there should be an X2A egg with a Y chromosome. Thus for each segregation type listed in Table 4 the percentage of eggs with a Y chromosome should add up to 100, but for the $\hat{X}\hat{X}$1A-X2A segregation there are marked deficiencies of 8–12 percent. Assuming that mortality of $\hat{X}\hat{X}$2A zygotes receiving the maternal and paternal Y chromosomes is the principal cause of the discrepancy, one can calculate that a mortality rate for such zygotes of 48–62 percent, respectively would account for the observed difference. Explanations based on preferential orientation of the first meiotic division are more complicated. One can account for the low number of attached-X diploid females by assuming that the presence of the Y chromosome causes the X2A pole to become the future egg nucleus in preference to the $\hat{X}\hat{X}$1A pole, but to explain the distribution of the Y chromosome one would have to assume that the preferential orientation is much more marked when the X2A pole does not receive the Y chromosome.

TABLE 4

Y chromosome segregation in attached-X triploid females

Triploid type	Chromosome constitution of eggs and recovered zygotes: Regular X chromosome segregations: X1A — $\hat{X}\hat{X}$2A: XX2A	X2A	$\hat{X}\hat{X}$X3A	$\hat{X}\hat{X}$3A	Regular X chromosome segregations: $\hat{X}\hat{X}$1A — X2A: $\hat{X}\hat{X}$X2A	$\hat{X}\hat{X}$2A	XX3A	X3A	Nondisj. X chromosome segregations: 01A — $\hat{X}\hat{X}$X2A: X2A	$\hat{X}\hat{X}$XX3A	$\hat{X}\hat{X}$X3A	$\hat{X}\hat{X}$1A — 02A: $\hat{X}\hat{X}$X2A	X3A	Total
$\hat{X}\hat{X}$,X,*sc*^{s}·Y														
Y present	394	338	17	6	..	243	35	3	33	..	..	1	7	1077
Y absent	24	19	366	198	2	53	328	25	..	7	17	1	2	1042
Percent with Y	94	95	4	3	..	82	10	11	100	0	0	..	78	51
$\hat{X}\hat{X}$,FM4,*sc*^{s}·Y														
Y present	186	118	12	2	..	91	10	..	40	..	..	..	15	474
Y absent	22	13	160	75	..	20	167	1	..	8	23	..	..	489
Percent with Y	89	90	7	3	..	82	6	..	100	0	0	..	100	49

TABLE 5

Frequencies of autosomally balanced and unbalanced eggs produced by attached-X triploid females

Type of triploid	Number of eggs counted	Number of recovered diploid zygotes: XX2A	XY2A	$\hat{X}\hat{X}$Y2A	Total	Percent of* autosomally balanced eggs recovered as 2N offspring	Percent of eggs with balanced sets of autosomes	Percent of eggs with unbalanced sets of autosomes
1. $\hat{X}\hat{X}$,X	1960	60	51	80	191	30–36	32–27	68–73
Percent of eggs		3.0	2.6	4.0	9.6			
2. $\hat{X}\hat{X}$,FM4	2809	63	59	87	209	11–24	67–31	33–69
Percent of eggs		2.2	2.1	3.1	7.4			

* These percents are derived from the values shown in Table 3.

Analysis of autosomally unbalanced eggs

Proportion of balanced versus unbalanced eggs produced: Autosomally unbalanced eggs produced by triploid females ordinarily lead to lethal aneuploid zygotes. The total proportion of such eggs can be estimated by making egg counts and determining from the diploid offspring produced what the total number of autosomally balanced eggs should be, the remainder being the unbalanced eggs. The ratio of diploid offspring to total number of autosomally balanced eggs can be found by using the frequencies of the different segregation types recorded in Table 3. The results of such egg counts and calculations are shown in Table 5. They indicate that about 70 percent of the eggs from attached-X triploid females with a normal free X chromosome are unbalanced autosomally. A certain proportion of the eggs which would have been recovered as diploid offspring probably die for unknown causes and thus contribute to the total pool of "unbalanced" eggs. Because the number of diploid offspring produced is such a small percentage of the eggs laid, the degree of such unknown mortality would have to be very great to significantly affect the 70 percent. For example, if only 90 percent of the potential diploid offspring survived, the frequency of diploid zygotes recovered would be ten percent too low and the calculated 30 percent of eggs with balanced sets of autosomes would be ten percent too low. This would only lower the proportion of autosomally unbalanced eggs to 67 percent.

Because of the uncertainty regarding the viability of metamales the proportion of unbalanced eggs formed by triploid females with the FM4 chromosome could be anywhere from 33–69 percent. There is good reason to believe that the 33 percent figure is too low. As discussed earlier in connection with Table 3 the viability of regular metamales carrying the FM4 chromosome, on which the 33 percent figure is based, was probably too low an estimate of the viability of the exceptional metamales. A direct test of the frequency of unbalanced eggs is also possible by comparing the two types of triploids shown in Table 5 with respect to the frequency with which nondisjunctional sperm are recovered from the same types of males. FROST's data (1961, Table 2, Experiments I, II and III) are here analyzed in this regard. For such a comparison the number of attached-X diploid females produced can be used as a measure of the size of the experiment. From the FM4 type of triploid, 14 nullo II sperm were recovered out of 11,045 attached-X diploid females produced, from the triploids carrying a normal free X chromosome the numbers were 11 out of 8,752 respectively, the rate for both types of triploids being 0.13 percent. This does not necessarily mean that both types of triploid females form the same proportion of unbalanced eggs since Table 5 shows that per egg laid, triploid females with the FM4 chromosome produced only 77 percent as many attached-X diploid females. Thus for an identical rate of autosomally unbalanced eggs produced per attached-X diploid female, the total proportion of unbalanced eggs produced by the FM4 triploid females need only be 52–56 percent.

For any one class of diploid offspring listed in Table 5 the difference in proportion of diploid zygotes recovered between the two types of triploid females is

not significant at the five percent level. For the total number of diploid offspring produced, however, the difference in proportion is significant at the one percent level.

Different types of autosomally unbalanced eggs formed by triploid females: Autosomally unbalanced eggs can be recovered if they are fertilized by the complementary type of unbalanced sperm, or by sperm where the subsequent loss of a paternal chromosome in the developing zygote mimics fertilization by nullo II or nullo III sperm. The latter effect can be accomplished by X-irradiation of sperm and extensive data of this sort are given by PONTECORVO (1943). The former type of sperm result from autosomal nondisjunction in the male, a phenomenon described by STERN (1934), PONTECORVO (1940), and FROST (1961), and that gives rise to diplo II and diplo III sperm as well as nullo II and nullo III sperm. Thus eggs with a II;III,III constitution have been recovered by being fertilized with diplo II sperm (forming intersexes or triploid females) or with nullo III sperm (forming diploid males or females). Eggs with the II.II;III constitution have been recovered in similar fashion with nullo II and diplo III sperm.

PONTECORVO (1943) pointed out that $X\hat{X}$;II,II;III–X;II;III,III segregations were just as frequent as X;II,II;III–$X\hat{X}$;II;III,III segregations, i.e. that sex chromosome orientation with respect to the autosomes was random. He also found the same to be true when nondisjunction of the sex chromosomes had occurred. The frequency of nondisjunction was high. Of the 140 exceptions he recovered in which one paternal autosome had been lost, 22 were patroclinous males. However it is not possible from his data to disentangle the various factors leading to sex chromosomal nondisjunction. The triploid females used by PONTECORVO carried attached-X chromosomes and a free X chromosome carrying two inversions, and about 60 percent of the females also carried a Y chromosome.

Table 6 shows how the autosomal exceptions reported by FROST (1961, Table 2) were broken down with respect to the egg types recovered. It pools data obtained from attached-X triploid females with a normal free X chromosome as well as from triploid females with the FM4 chromosome. Since any given line in Table 6 represents just one type of nondisjunctional sperm, the number of different egg types recorded in that line reflects their relative frequency. Allowance must be made, of course, for the lower viability of intersexes or triploid metafemales. Thus data for sperm types no. 2, no. 3, no. 6, and no. 7 accord with PONTECORVO's observation that free X and attached-X chromosomes occur with equal frequency in both types of autosomally unbalanced eggs. The data for sperm types no. 1 and no. 5 show that nondisjunction of the X chromosomes is quite frequent, four out of the 18 eggs (22 percent) recovered were nullo X. The occurrence of triplo X eggs is also demonstrated in the line for sperm type no. 8. Of the 18 eggs recovered with sperm types no. 1 and no. 5, 11 came from triploid females with the normal free X chromosome, two being nullo X, the proportion of nondisjunctional eggs for the FM4 triploid females being two out of seven. Consequently the frequency of sex chromosomal nondisjunction is relatively high for both types of females. It should be noted that the data for sperm type no. 5 of

TABLE 6

*X chromosome constitution of autosomally unbalanced eggs produced by attached-X triploid females**

Constitution of sperm	Constitution of egg II,II;III 0	X	$\widehat{XX}$	$\widehat{XX}$,X	Sex types of offspring
Nullo II sperm					
1. X bearing	3	8	—	—	diploid male and female
2. Y bearing	—	6	8	—	diploid male and female
Diplo III sperm					
3. X bearing	—	3	3	—	intersex and triploid female
4. Y bearing	—	—	0	0	intersex and triploid female
	II;III,III				
Nullo III sperm					
5. X bearing	1	6	—	—	diploid male and female
6. Y bearing	—	5	5	—	diploid male and female
Diplo II sperm					
7. X bearing	—	0	4	0	intersex, triploid female, and triploid metafemale
8. Y bearing	—	—	0	1	intersex and triploid female

* This table represents a further analysis of the information on autosomal nondisjunction in males recorded in Table 2 of FROST 1961.

Table 6 came from an experiment where the possibility of the triploid female parents carrying a Y chromosome had not yet been excluded. However, judging from the frequency of exceptional diploid males, the proportion of such females, if they occurred, must have been very small.

The two types of unbalanced eggs discussed so far are not the only ones produced by triploid females. In crosses with triploid females having three free X chromosomes STERN (1934) found nullo II and nullo III eggs (X;0;III and X;II;0 respectively), and FROST (1961) also reported the occurrence of a nullo II egg (X;0;III) from an attached-X triploid female. These egg types were recovered when fertilized by diplo II or diplo III sperm, thus forming diploid offspring. To gain an estimate of the relative frequency of nullo II eggs we can compare them with II,II;III eggs which also result in diploid offspring if fertilized by nullo II sperm. Assuming that nullo II and diplo II sperm occur equally often, as indicated by the results of FROST (1961), the two different egg types would be recovered with equal frequency if they occurred with equal frequency. The same type of comparison can be made for nullo III eggs and II;III,III eggs. In FROST's experiments only one 0;III egg was recovered compared to 25 II,II;III eggs. In STERN's experiment the ratio was seven to six respectively for chromosome II, and for chromosome III the ratio was five to two. The higher frequency of nullo-type eggs observed in STERN's experiments may reflect the influence of the different structure of the X chromosomes present in the triploid females used.

DISCUSSION

BEADLE (1935) suggested that as a result of the mass and number of chromosomes gathered together at metaphase they would tend to become oriented so that both sides of the metaphase plate would have equal numbers of chromosomes, a tendency termed the crowding effect by SANDLER and NOVITSKI (1956). In this way BEADLE explained why $\hat{XX}$1A-X2A segregations were more common than X1A-$\hat{XX}$2A segregations, and why $\hat{XX}$X1A-02A segregations were more common than 01A-$\hat{XX}$X2A segregations. The present paper shows that the segregation of the Y chromosome follows the same rule, as it is most often recovered at the pole with the fewest chromosomes.

The possibility that chromocentral regions of non-homologous chromosomes might pair and subsequently disjoin was suggested by MULLER (cited by PONTECORVO 1940) as an explanation for the preferential segregations observed in triploid females. This idea did not receive further attention until it was proposed anew in more detail by SANDLER and NOVITSKI in 1956. The latter two authors reviewed evidence for considering heterochromatic regions of the chromosomes to be nonspecific in their pairing affinities, and suggested that if each set of homologues in a triploid formed a bivalent plus a univalent, non-homologous pairs could easily form between the heterochromatic kinetochore regions of the different univalents. They pointed out that pairing between chromosome II and III would result in unbalanced eggs and that pairing between an X chromosome and an autosome would result in the preferential recovery of eggs from the XX1A-X2A segregation. They suggested that in attached-X triploid females the free X chromosome and attached-X chromosomes would often form what was essentially a single bivalent, thus decreasing opportunities for non-homologous pairing.

About the same time that SANDLER and NOVITSKI suggested the above hypothesis a similar proposal was made with respect to interchromosomal effects on segregation in diploid females. COOPER, ZIMMERING, and KRIVSHENKO (1955) suggested that non-homologous pairing was responsible for the increased frequency of primary nondisjunction of the X chromosomes observed when both the X chromosomes and an autosome were heterozygous for inversions. These authors, however, remained noncommittal about the chromosome regions involved in the non-homologous association.

Much of the new information on triploid segregations presented here can be readily explained by the non-homologous pairing hypothesis of SANDLER and NOVITSKI.

In the discussion which follows it will be assumed that the pairing tendencies of the attached-X chromosomes are never entirely "self-satisfying" as indicated, for example, by the fact that the Y chromosome and attached-X chromosomes in diploid females regularly segregate to opposite poles. Consequently, whenever the attached-X chromosomes and free X chromosome have not paired with each other in triploid females, both chromosomes are considered to be available for non-homologous association. Pairing may not always ensure disjunction but for

the purposes of the present discussion the terms "pairing" and "association" are used in a restricted sense to indicate only those cases in which the pairing does ensure the disjunction of the two chromosomes involved.

That 70 percent of the eggs produced by attached-X triploid females with a normal free X chromosome are unbalanced autosomally can be accounted for by pairing between chromosomes II and III. Such pairing always results in unbalanced eggs. If the "extra" chromosomes II and III segregated randomly with respect to each other one would expect only 50 percent of the segregations to result in unbalanced eggs. The excess over 50 percent could be accounted for if 40 percent of the segregations were preceded by II-III pairing, half of the remaining segregations also producing unbalanced eggs.

There are two ways in which the "extra" chromosomes II and III could be expected to segregate randomly with respect to each other, (1) if all associations were homologous and the two autosomal trivalents oriented randomly at the metaphase plate, and (2) if non-homologous associations formed between an autosome and a sex chromosome, the resulting non-homologous pair orienting randomly with respect to the other chromosomes. This second way suggests that if the sex chromosomes were made more available for non-homologous pairing X-chromosome-autosome pairs should form at the expense of II-III pairs and the total frequency of autosomally unbalanced eggs should drop from 70 percent to a value closer to 50 percent. Such appears to be the case for the triploid females carrying the FM4 chromosome. The multiple inversions present in the free X chromosome would make both the attached-X chromosomes and the free X chromosome available for non-homologous pairing.

Practically all of the X chromosome nondisjunction occurring in the FM4 triploid females can be attributed to X-chromosome-autosome pairing since the frequency of 01A-$\hat{X}\hat{X}$X2A segregations is negligible compared to the frequency of $\hat{X}\hat{X}$X1A-02A segregations. The former type of segregation would never occur as a result of X-chromosome-autosome pairing but should be as frequent as $\hat{X}\hat{X}$X1A-02A segregations were the X chromosomes segregating randomly with respect to the autosomes. If we take 54 percent as the best estimate of the frequency of autosomally unbalanced eggs formed by the FM4 triploid females, then autosomally balanced eggs account for 46 percent of the segregations and close to half of these balanced eggs would result from the $\hat{X}\hat{X}$X1A-02A segregation.

The introduction of a Y chromosome causes a marked increase in the frequency of 01A-$\hat{X}\hat{X}$X2A segregations occurring in the FM4 triploid females. Since nondisjunction of the X chromosomes can result from X-Y pairing, and since the latter type of pairing reduces the number of X chromosomes available for nonhomologous pairing, a portion of the segregations resulting in X chromosome nondisjunction could be expected to show independent orientation of sex chromosomes and autosomes. It is not contradictory to the above reasoning to realize that pairing of the Y chromosome with an X chromosome also makes the remaining X chromosome available for non-homologous pairing. Thus in examining the

effect of the Y chromosome on segregation in attached-X triploid females with a normal free X chromosome, the increase in nondisjunction frequency is accompanied by the same marked preference for the $\widehat{XX}$X1A-02A segregation. The $\widehat{XX}$X1A-02A segregations were 3–6 times more frequent than 01A-$\widehat{XX}$X2A segregations but for every one of the latter segregations there should be one of the former type where no X autosome association was involved either. Consequently anywhere from 50–29 percent respectively of the segregations resulting in X chromosome nondisjunction involved independent orientation of sex chromosomes and autosomes. One could imagine that the X chromosomes would be least available for non-homologous pairing when the Y pairs with both the free X chromosome and an arm of the attached-X chromosome.

An examination of the consequences of X-Y pairing makes it seem unlikely that the Y chromosome regularly pairs with the X chromosomes. It should be possible for the attached-X chromosomes to pair with the free X chromosome and the Y chromosome. Such pairing would result in regular disjunction of the X chromosomes but cannot be very frequent since there is no indication that the Y chromosome segregates preferentially from the attached-X chromosome. The Y chromosome could pair with both the free X chromosome and an arm of the attached-X chromosomes, or it could pair with either of the X chromosomes separately. The former situation would always result in X chromosome nondisjunction and the latter situation would result in X chromosome nondisjunction 50 percent of the time since the X chromosomes would segregate randomly with respect to each other. For the triploid females with the normal free X chromosome, at least, the nondisjunctional segregations account for only 20–30 percent of the autosomally balanced eggs (see Table 3). Consequently the Y chromosome, at most, would only be paired with the X chromosomes in 40–60 percent of the segregations producing autosomally balanced eggs.

To explain the preferential recovery of the Y chromosome at the 1A pole one must make one or both of the following assumptions, depending on the frequency of X-Y pairing: (1) when the Y chromosome had not paired with an X chromosome, it usually had paired with an autosome, or (2) when the Y chromosome had paired with one of the X chromosomes, the remaining X chromosome usually had paired with an autosome. Since the Y chromosome was recovered at the 2A pole about ten percent of the time, in only 20 percent of the regular X chromosome segregations would it not be necessary to invoke one of the above assumptions.

Attached-X triploid females show striking differences from free X diploid females with respect to frequencies of X chromosome nondisjunction. Primary nondisjunction in diploid females occurs with a very low frequency, less than 0.1 percent (Bridges 1916), and is affected very little by the presence of structural heterozygosity in the X chromosomes (Sturtevant and Beadle 1936). In attached-X triploid females, however, primary nondisjunction certainly occurs with a frequency greater than 0.1 percent, more probably with a frequency of several percent, and the introduction of the FM4 free X chromosome causes high

frequencies of nondisjunction. Frequencies of secondary nondisjunction parallel the differences observed in primary nondisjunction. In diploid females with normal X chromosomes the frequency of secondary nondisjunction is about eight percent (BRIDGES 1916), but the introduction of a Y chromosome into attached-X triploid females produces frequencies of nondisjunction of 20–30 percent. The results of such comparisons, as well as the different types of preferential segregations shown by triploid females, agree well with the view that in triploid females meiotic pairing is by twos, and that one member of any given set of homologous chromosomes is usually available for non-homologous associations.

Triploid metafemales (4X3A) provide one further test of this view of chromosome segregation in triploids. In triploid metafemales carrying attached-X chromosomes, one normal free X chromosome, and the FM4 chromosome, FROST (1960) reported that most of the attached-X chromosomes recovered came from 2A eggs, and that most of the free X chromosomes recovered came from 1A eggs. This type of preferential segregation is the opposite of that found in attached-X triploid females (see Table 2 and BEADLE 1934, 1935). The attached-X chromosomes and the normal free X chromosome would be expected to pair most of the time but the FM4 chromosome should usually be available for non-homologous associations. Thus the attached-X chromosome and the normal free X chromosome should usually separate from each other randomly with respect to the autosomes, but the FM4 chromosome should be found preferentially at the 1A pole. The resulting segregations would be of two kinds, FM4$\hat{X}\hat{X}$1A-X2A and FM4X1A-$\hat{X}\hat{X}$2A. The latter type of segregation produces the observed result. The only offspring recoverable from the former type of segregation would be metamales, intersexes, or diploid metafemales. None were recovered but this is not significant since only one of the 16 2X2A eggs recorded was recovered as an intersex. This explanation would predict that in triploid metafemales carrying two normal free X chromosomes this type of preferential segregation would disappear. If both free X chromosomes paired with the attached-X chromosome, the resulting complex should orient randomly with respect to the autosomes. If the two free X chromosomes paired with each other, the attached-X chromosomes should be available for non-homologous pairing and $\hat{X}\hat{X}$X1A-X2A segregations would be more frequent than $\hat{X}\hat{X}$X2A-X1A segregations. However the latter type would be recovered preferentially since the former type leads to offspring with very poor viability.

There were two observations which violated the crowding effect rule. The first is compatible with the analysis made up to this point and concerns the apparent high frequency of X chromosome nondisjunction among the autosomally unbalanced eggs formed by attached-X triploid females with the normal free X chromosome. One would expect that frequencies of X chromosome nondisjunction in autosomally unbalanced eggs should be the same, or even less than the frequencies observed in autosomally balanced eggs. For example, if both the attached-X chromosomes and the free X chromosome had formed separate nonhomologous pairs with different autosomes, segregation of the two pairs would

result either in autosomally balanced eggs but nondisjunction of the X chromosomes, or in autosomally unbalanced eggs but regular disjunction of the X chromosomes. If only one non-homologous pair formed and the remaining sex chromosome and autosome segregated randomly, nondisjunction of the X chromosomes should occur equally often in both autosomally balanced and unbalanced eggs. Table 3 indicates that the frequency of X chromosome nondisjunction among the autosomally balanced eggs is 1–17 percent. The observation of two nondisjunctional eggs out of 11 autosomally unbalanced eggs suggests that the true frequency of nondisjunction in the former case lies somewhere in the middle or upper part of the range.

The second violation of the crowding effect rule concerns the existence of X;0;III or X;II;0 eggs. At present there appears to be no adequate explanation for the occurrence of such eggs. The non-homologous pairing hypothesis does not provide an adequate explanation since it is difficult to see how so few chromosomes could determine the disjunction of so many other chromosomes to the opposite pole. The presence of undetected autosomal inversions might provide a possible mechanism for the production of such eggs. Four strand double crossovers within heterozygous X chromosome inversions are known to result in nullo X eggs (STURTEVANT and BEADLE 1936). A similar type of double crossover occurring within a paracentric autosomal inversion might prevent the two chromosomes involved from reaching the future egg nucleus if the chromosome bridges did not break. The remaining chromosome could be expected to segregate to the future egg nucleus pole or to the future polar nucleus pole at random. In order to explain STERN's (1934) results on this basis one would not only have to assume that both chromosome II and III were carrying inversions but that the frequencies of four strand doubles were enormously high. Nullo type eggs were recovered as frequently as II,II;III or II;III,III eggs and it seems unlikely that triploid females produce autosomally unbalanced eggs with a frequency lower than 50 percent.

While the present information on segregations in triploid females provides abundant evidence for interactions between sex chromosomes and autosomes, it does not indicate whether there are preferential associations between particular chromosomes. From a study of autosomally balanced eggs, for example, it is not possible to tell whether the sex chromosomes had paired with chromosome II or III, even if both were "marked" by the use of multiple inversions. If a sex chromosome had paired with the marked chromosome II, then the only eggs that would be recovered would be those in which the marked chromosome III had also proceeded to the pole opposite the sex chromosome. Studies of autosomally unbalanced eggs should provide answers to this question but our present information is rather meagre. PONTECORVO's results would indicate that non-homologous pairs do not form exclusively between only one of the autosomes and the sex chromosomes, but it is difficult to know just how much non-homologous pairing would have been expected in the triploid females he used. The most rewarding studies would be on free X triploid females, or on attached-X triploid females

with the FM4 chromosome where the majority of the segregations probably involve non-homologous pairing between the X chromosomes and autosomes.

SUMMARY

Evidence is presented which confirms BEADLE's (1934, 1935) findings that primary nondisjunction of the X chromosomes occurs with an appreciable frequency in attached-X triploid females, that the segregation of the autosomes is highly non random when X chromosome nondisjunction occurs such that 01A-$\hat{X}\hat{X}$X2A segregations occur with a negligible frequency compared to $\hat{X}\hat{X}$X1A-02A segregations, and that the presence of a Y chromosome produces large increases in X chromosome nondisjunction. Attached-X triploid females have been further studied in relation to the following points:

1. The presence of a free X chromosome carrying multiple inversions causes large increases in the frequency of primary nondisjunction. The increase is accompanied by the same preferential orientation of the autosomes.

2. The presence of a Y chromosome causes substantial increases in the frequency of 01A-$\hat{X}\hat{X}$X2A segregations, but $\hat{X}\hat{X}$X1A-02A segregations are still 3–6 times more frequent.

3. When regular disjunction of the X chromosomes takes place, the Y chromosome is almost always recovered at the pole with one set of autosomes. When nondisjunction of the X chromosomes occurs, the Y chromosome is almost always recovered at the nullo X pole.

4. The number of regular diploid offspring recovered from triploid females with a normal free X chromosome or a free X chromosome carrying multiple inversions, accounts for only 9.6 percent and 7.4 percent respectively of the total number of eggs produced. The corresponding estimates of the total frequency of autosomally balanced eggs produced by the two types of triploids are 27–32 percent and 31–67 percent respectively.

5. Further evidence is given supporting PONTECORVO's (1941) observation that there is no preferential segregation of the X chromosomes with respect to the autosomes among autosomally unbalanced eggs.

6. A coherent explanation of all the above known facts concerning segregation in triploid females can be made on the basis of SANDLER and NOVITSKI's (1956) hypothesis that pairing can frequently occur between non-homologous univalents in their heterochromatic kinetochore regions. However there is no adequate explanation as yet for the occurrence of nullo II and nullo III eggs reported by STERN (1934) and FROST (1961).

7. The sharp reduction in the number of attached-X diploid females produced by triploid females carrying a Y chromosome is probably caused by low viability of attached-X females receiving both the maternal and paternal Y chromosomes.

8. Age of the triploid female, up to 15 days, has no effect on the frequency of X chromosome nondisjunction or other patterns of chromosome segregation.

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CHLOROPHYLL LETHAL IN NATURAL POPULATIONS OF THE ORCHARD GRASS (DACTYLIS GLOMERATA L.). A CASE OF BALANCED POLYMORPHISM IN PLANTS[1,2]

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IN recent years extensive information has been gathered as to the occurrence and evolutionary significance of recessive lethal genes in natural panmictic populations (for details see reviews by DOBZHANSKY 1951, 1955, 1959). Work has concentrated almost exclusively on several Drosophila species, undoubtedly due to the fact that extraction techniques in this genetically well-studied genus are highly developed. Strikingly high frequencies of recessive lethals have been found to be a common feature in natural populations of various Drosophila species, and a model of balanced polymorphism has been suggested to account for these facts.

Compared to the genus Drosophila, information concerning the occurrence and distribution of recessive lethals in other organisms is rather meagre. An exception is the case of man; here high frequencies of the sickle cell factor in some human populations (ALLISON 1954, 1956) as well as those of several other deleterious recessive genes (NEEL 1956), have been explained on the basis of a model of balanced polymorphism.

In the present paper data are brought forward which indicate a case of balanced polymorphism, involving a recessive lethal in plants. It deals with the occurrence and distribution of a chlorophyll-deficiency factor in natural populations of diploid ($2n = 14$) orchard grass (*Dactylis glomerata*) in Israel. The study was initiated after it had been noted that certain seed samples collected for breeding purposes from individual wild Dactylis plants in the vicinity of Jerusalem, yielded a high proportion of white, chlorophyll-deficient seedlings (up to 12–15 percent). Since Dactylis is self-incompatible, such progeny should have originated from cross-pollination of a heterozygous parent by pollen carrying the mutant gene. On the assumption that an albino seedling is homozygous for a single recessive lethal locus, the appearance of up to 12–15 percent of albino seedlings among progeny of a given plant seemed to indicate that chlorophyll-deficiency genes were widely distributed in the local Dactylis populations.

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[2] The data presented are taken from the M.S. thesis submitted by the first author to the Dept. of Botany, The Hebrew University, Jerusalem.

MATERIAL

Both diploid ($2n = 14$) and tetraploid ($2n = 28$) forms of *Dactylis glomerata* L. occur in Israel (for details on their geographical distribution see NUR and ZOHARY 1959). The present work deals only with natural populations of the local diploid form: *D. glomerata* ssp. *judaica*. Tetraploids have not been investigated.

D. glomerata ssp. *judaica* occurs in Israel mainly in three areas: (a) the Judaean Hills around Jerusalem, (b) Nazareth Hills, (c) the area of Safad, Upper Galilee. Here, occurring on several different soil types, this perennial grass constitutes a common component of the dwarf shrub and herbaceous plant communities (Mediterranean "batha" formations) which are a characteristic vegetational feature of these areas.

As to its breeding system, *Dactylis glomerata* ssp. *judaica* is a truly panmictic or cross-pollinated plant. Seed is produced sexually, and the plants are entirely self-incompatible. The latter fact has been verified by the present authors in numerous breeding experiments. No seed has ever been obtained upon the selfing of local Dactylis plants.

EXPERIMENTAL PROCEDURE AND RESULTS

The frequencies of chlorophyll-deficiency genes have been investigated in 14 samples taken from 13 chosen localities occupied by natural populations of *Dactylis glomerata* ssp. *judaica*. They are representatives of the main areas in which diploid orchard grass is known to occur in Israel and constitute a cross section of the various Israeli habitats occupied by this grass.

Sampling and seed harvesting were carried out at the end of the growing season, in July 1958 (8100-series) and in June 1959 (9100-series). Sampling procedures were as follows: At each station 100 plants were chosen for examination of their progeny, and seed from each plant was harvested separately in the field. Plants were sampled at each station along three or four transects which were 15–20 meters apart. Plants were sampled in pairs. The distance between pairs was 5–10 meters, within pairs 1–2 meters. The sampling of pairs of adjacent plants was employed to check on homogeneity of spread of heterozygotes in the examined areas. This was checked by means of a binomial test, the two events being identical pairs (i.e. both partners homozygous normal or both heterozygous) *versus* heterogeneous pairs (i.e. one partner homozygous normal, the second heterozygous). These tests indicated that the homozygous and heterozygous plants were distributed at random, with no local concentrations of one or the other genotype. There was no indication of uneven spread in the populations sampled.

Estimates of the frequencies of chlorophyll-deficiency genes in the various populations were obtained in two ways: (a) heterozygous plants were detected by means of progeny tests; (b) plants whose heterozygosity had been established were used as natural testers.

Detection of heterozygous plants by means of progeny tests: The seed sample collected from each respective plant was sown in the winter following harvesting

under greenhouse conditions in Jerusalem. The germinating seedlings were examined 3–4 weeks after sowing. The number of green (or normal) and albino (or chlorophyll-deficient) progeny obtained from each plant was recorded. A plant was assumed to be heterozygous for a chlorophyll-deficiency factor, when there was at least one albino seedling among its progeny.

The number of plants, in each locality, heterozygous for a chlorophyll-deficiency recessive, as detected by means of progeny tests is given in Table 1.

Naturally, the numbers of seeds that could be harvested from the individual parents varied greatly from plant to plant and from locality to locality. An effort was made to secure at least 50 seeds from each plant, but this was not possible in every case. Individuals which yielded less than ten seedlings were discarded from

TABLE 1

Detection of heterozygous plants: Results of progeny tests

Sample number	Locality and soil type	Total number of plants tested	Number of normal homozygous plants	Number of heterozygous plants	Percent of heterozygotes in sample	Estimated gametic frequency of albino gene (percent)
Jerusalem Hills:						
DC 8101:	Matzleva Valley terra-rossa	47	35	12	26	13
DC 8102:	Ora rendzina	79	65	14	18	9
DC 8103:	Aminadav rendzina	94	81	13	14	7
DC 8104:	Schneller Camp terra-rossa	78	55	23	29	14.5
DC 8105:	University campus terra-rossa	64	58	6	9	4.5
DC 8106:	Qiryat Anavim terra-rossa	80	66	24	30	15
DC 9101:	Qiryat Anavim terra-rossa	94	66	28	30	15
DC 9102:	Kastel terra-rossa	100	72	28	28	14
Nazareth Hills:						
DC 8110:	Kefar Hahoresh rendzina	82	79	3	4	2
DC 8111:	Nazareth rendzina	95	90	5	5	2.5
Upper Galilee:						
DC 8107:	Ein Zeitim rendzina	79	76	3	4	2
DC 8108:	Safad rendzina	88	77	11	13	6.5
DC 8109:	Biriya mixed soil	86	76	10	12	6
DC 8112:	Miar rendzina	97	82	15	15	7.5

further considerations, whether they produced only green—or both normal and albino seedlings. This is the cause for the discrepancy between the 100 plants originally sampled at each locality and the results of the progeny tests presented in Table 1.

In some stations and particularly in the Jerusalem area, strikingly high percentages (26–30 percent) of plants yielded albino seedlings, thus indicating a large proportion of heterozygotes. It is probably safe to regard the plants which yielded albino progeny as heterozygous for a recessive chlorophyll-determining factor. But it is obviously impossible, solely by means of that method, to decide whether these plants are heterozygous for the same deficiency gene, or whether they represent several chlorophyll-determining factors at different loci. Yet the values obtained from utilization of heterozygotes as natural testers (see next section) strongly indicate that, at least within a respective locality, most of the plants which had yielded albino seedlings were actually heterozygous for the same recessive gene. The estimates of the gametic frequencies given in Table 1 were therefore calculated on the basis of such an assumption.

Heterozygotes as natural testers: Since plants of this taxon are self-incompatible and necessarily cross-pollinated, any individual whose heterozygosity had been established could be used as a natural tester for estimating the proportion of pollen, carrying the albino gene, produced in its vicinity. Any such pollen grain germinating on the stigma of a heterozygous plant has a 50 percent chance to produce a homozygous albino progeny. Assuming that there is no selective elimination (either in gametes or in zygotes), the percentage of albino seedlings in the progeny of a given heterozygote thus represents one half of the gametic frequency of the chlorophyll-deficient lethal and one fourth of the percentage of heterozygous plants within the sampled area.

In each locality, the data on progeny of plants which yielded albino seedlings (and were therefore assumed to be heterozygous) were also utilized for such an estimate of the gametic frequency of the chlorophyll-deficiency gene. To avoid complications, only heterozygotes which yielded 30 seedlings or more were included in these estimates. Table 2 summarizes the estimates of the gametic frequencies of the chlorophyll-lethal obtained upon utilization of the heterozygotes in each locality as natural testers.

In the majority of stations these estimates agree quite closely with the percentages of heterozygous plants as detected by means of the first method (compare Tables 1 and 2). The only major exception is sample DC 8105, where the values based on natural testers are exceptionally high and disagree entirely with the low number of detected heterozygotes. This general agreement between the two sets of data strongly suggests that at least within a given locality one is dealing mainly with a single lethal gene. Obviously, if several different factors for chlorophyll-deficiency had been present in a respective population, the frequency values as calculated from the testers (Table 2) would have been considerably lower than those based on detected heterozygotes (Table 1).

Ecological differences: The Dactylis populations examined were growing on two main types of soils. Some localities were characterized by typical mediter-

ranean red-soil or "terra rossa". This soil type develops on a hard limestone and dolomite bed-rock. In other localities the soil was a light colored rendzina. This is a highly calcareous soil which is formed, in the mediterranean areas of Israel, on soft marls and chalks. Only in one case (DC 8109) the sample was collected in a place where the two soil types formed a mosaic. The soil type of each locality is noted in Table 1 and 2. Further ecological details on each sample may be obtained by any interested reader, by writing to the junior author.

As can be seen from the data set out in Tables 1 and 2, the frequency of the chlorophyll-blocking gene is much higher in Dactylis populations occupying terra rossa than in those occupying rendzina soil. In the Jerusalem area these

TABLE 2

Frequencies of the chlorophyll-deficiency gene as estimated by use of heterozygotes as natural testers

Sample number	Locality and soil type	Number of testers employed	Total number of green progeny	Total no. of chlorophyll deficient progeny	Total number of progeny	Estimated gametic frequency (percent)	Estimated frequency of heterozygotes (percent)
Judaean Hills:							
DC 8101:	Matzleva Valley terra-rossa	7	375	25	400	12.5	25
DC 8102:	Ora rendzina	12	868	22	890	5	10
DC 8103:	Aminadav rendzina	13	1511	32	1543	4	8
DC 8104:	Schneller Camp terra-rossa	21	2069	145	2214	13	26
DC 8105:	University campus terra-rossa	5	318	43	361	24	48
DC 8106:	Qiryat Anavim terra-rossa	21	1448	95	1543	13	26
DC 9101:	Qiryat Anavim terra-rossa	20	2290	111	2401	9.5	19
DC 9102:	Kastel terra-rossa	18	3795	167	3962	8.5	17
Nazareth Hills:							
DC 8110:	Kefar Hahoresh rendzina	3	160	3	163	3.5	7
DC 8111:	Nazareth rendzina	5	432	5	437	2.5	5
Upper Galilee:							
DC 8107:	Ein Zeitim rendzina	3	420	7	427	3.5	7
DC 8108:	Safad rendzina	11	733	28	761	7.5	15
DC 8109:	Biriya mixed soil	10	718	16	734	4.5	9
DC 8112:	Miar rendzina	15	2995	70	3065	4.5	9

differences are highly indicative, since stations on different soil types are geographically in close proximity (distances of 3–4 km), and the Dactylis cover of this area is more or less continuous.

DISCUSSION

The most plausible explanation of the results obtained in the progeny tests is the occurrence of a recessive, chlorophyll-blocking allele in relatively high proportions in some natural populations of the diploid orchard grass in Israel. That one is dealing mainly with a single locus in each respective population is indicated by the agreement between the number of detected heterozygotes (Table 1) and the estimates based on natural testers (Table 2). Although the most likely situation is that of a simple Mendelian trait, this has yet to be verified. The final evaluation of the results obtained can be made only after genetic analysis has been carried out in at least some of the albino-producing plants, and after it has been confirmed that they are actually simple heterozygotes, and that one is dealing with the same locus in the various samples.

As to the detection technique employed, it is realized that some heterozygotes could have been missed, and erroneously considered as normal homozygotes, because a limited and variable number of progeny (ten to several hundreds) were raised from each tested plant. This is particularly true in populations where the frequency of the albino allele was found to be low. However, such errors would not change the general picture obtained.

The high proportions of the recessive lethal gene are best explained on the assumption of a higher selective value of heterozygous plants as compared to that of homozygous normal individuals. An equilibrium of adaptive polymorphism for a recessive lethal is proposed to account for the results obtained. This assumption of higher selective values of heterozygotes is further supported by the differences noted between populations occupying terra rossa and these growing on light colored rendzina soil, which indicate different selective values in different ecological niches.

Finally, attention should be drawn to the possibility that a chlorophyll-blocking class of mutations might be of general evolutionary significance in plants. The studies of WILLIAMS and BROWN (1956) on the cherry demonstrate that selection for quantitative economic traits has favored heterozygosity for chlorophyll-blocking recessives. Apparent vegetative vigor was found by GUSTAFSSON (1946) and GUSTAFSSON, NYBOM and VON WETTSTEIN (1950) in barley heterozygotes for induced, recessive chlorophyll mutations. The diploidization of the *Ws* locus in *Nicotiana tabacum* reported by CLAUSEN and CAMERON (1950) might be regarded as being of similar significance. The data presented in this paper indicate that such lethals can maintain themselves in a panmictic population. Adaptive polymorphism involving chlorophyll-deficiency factors is probably not restricted to the orchard grass alone. It may be of a more general occurrence in cross-pollinated plant species.

SUMMARY

Natural populations of diploid (2n = 14) orchard grass (*Dactylis glomerata*) native in Israel were examined as to the frequency of recessive chlorophyll-deficiency lethal in them. Frequencies up to 30 percent of heterozygous plants (yielding albino progeny) were found in some localities. It was also noted that populations growing on terra-rossa soils show higher proportions of the lethal gene than populations occupying rendzina soils.

The most likely explanations for the results obtained is that in certain habitats, heterozygotes for a chlorophyll-blocking lethal have a higher selective value than normal homozygotes. Apparently a condition of balanced polymorphism with regard to this locus is present in the populations of the orchard grass tested.

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NON RANDOM ASSOCIATION OF INVERSIONS IN DROSOPHILA PAVANI

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THE adaptive function of the chromosomal inversions found in many species of Drosophila is that of protecting coadapted complexes of polygenes. This is usually due to the fact that the chromatids which result from crossing over between relatively inverted gene sequences are not viable. The effects of inversions on genetic recombination are not restricted to the inverted regions of the chromosome; they may also strongly influence the frequency of recombination along the rest of it (STURTEVANT and BEADLE 1936; STEINBERG and FRASER 1944; DOBZHANSKY and EPLING 1948; SCHULTZ and REDFIELD 1951; CARSON 1953; LEVITAN 1954, 1955, 1958). Particularly interesting in this connection is the finding of non random association of linked gene arrangements in many species of Drosophila and related diptera. According to the review of LEVITAN (1958), the probable mechanism for maintaining such non random association of inversions would be natural selection which counteracts the effects of recombination, presumably because such crossovers disrupt the internally balanced gene systems carried by the chromosomes.

During the past several years data have been accumulated in our laboratory concerning the frequency and distribution of inversions in the chromosomes of *Drosophila pavani* Brncic 1957, a neotropical species which lives in Chile and in part of the Argentine. The study of several natural populations and laboratory stocks has led us to discover that the inversions in the left and right arms of the chromosome IV of *D. pavani* are often non randomly associated. Usually there is a significant overabundance of the "coupling" combinations, and a scarcity of the "repulsion" combinations of these gene orders. The data to be discussed in this paper seem to clarify well the role played by natural selection in the establishment of non random associations between linked inversions.

METHODS AND RESULTS

According to previous reports (BRNCIC 1957, 1958), *D. pavani* is a polymorphic species with respect to the gene orders in its chromosomes, due to the presence of inversions. Reference will only be made to those arrangements found in both arms of the fourth chromosome (Figure 1). In the middle part of the

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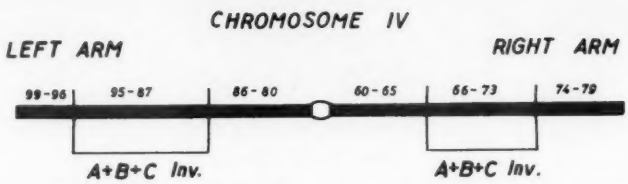

FIGURE 1.—Diagram of the relative length of the euchromatic sections of the salivary gland chromosome IV of *D. pavani*. The numbers above the dark line refer to the map regions (BRNCIC 1957). The inversions mentioned in this paper are bracketed. The light area in the center represents the heterochromatic base of the left and right arms included at the centromere.

left arm, and including about 45 percent of its length, there is a complicated gene sequence which differs from Standard by three overlapping inversions. Both Standard and this second type of arrangement (inversion IV-L; A+B+C in BRNCIC 1957) have been observed in all natural populations analyzed. The three inversions have never been found separately, neither in nature nor in laboratory stocks. On the right limb of this same chromosome, there is also in all populations studied another group of three overlapping inversions which occupy about 36 percent of the length (inversion IV-R; A+B+C in BRNCIC 1957). Like those on the left arm, none of these three inversions has ever been found alone. The different arrangements in both limbs of the fourth chromosome are very abundant in all the populations studied (BRNCIC 1957, 1958).

According to the mathematical analysis of the distribution of genes in populations (see LI 1955), the zygotic proportion with respect to two pairs of linked gene arrangements, in a random-mating equilibrium population, should be identical to that for independent inversions. In other words, the zygotic proportion should be determined exclusively by the frequency of each inversion, whether or not these are located in the same chromosome.

The data for testing whether the arrangements in the left and right arms of the fourth chromosome of *D. pavani* were associated at random or not, were obtained from the analysis of two types of samples: (1) male samples, and (2) egg samples.

(1) *Male samples:* Data concerning the gene sequences in the fourth chromosome in adult males were obtained from two stocks (Copiapó and Bellavista), maintained for several generations in the laboratory. These were used about 30 months after their establishment. Single males were mated to laboratory females homozygous for the "Standard" gene arrangement. The salivary gland cells of eight larvae from each cross were examined by the acetic orcein squash method in order to determine the gene structure of both homologous fourth chromosomes of the male. The observation of eight larvae renders the possibility of chance error negligible.

Table 1 shows that both in the Copiapó and Bellavista stocks there is an excess of fourth chromosomes carrying in both their left and right arms either "Standard" or the inverted gene arrangements, and a scarcity of recombination of such sequences. Chi-squares testing the goodness-of-fit of the observed data to the

TABLE 1

Observed (obs.) and expected (exp.) numbers of chromosome IV gene arrangements in adult males of D. pavani *from two laboratory stocks*

Chromosome IV		Copiapo stock		Bellavista stock	
Right arm	Left arm	obs.	exp.	obs.	exp.
st	st	71	28.8	135	66.8
st	Inv*	1	43.2	33	101.2
Inv†	st	9	51.2	24	92.2
Inv†	Inv*	119	76.8	208	139.8
Total:		200	200.0	400	400.0
Heterogeneity χ^2:			161.2		199.4
Probability:			<0.001		<0.001

* Corresponds to the IV-L (A+B+C) overlapping inversions in Brncic 1957.
† Corresponds to the IV-R (A+B+C) overlapping inversions in Brncic 1957.

corresponding expected values under the assumption of random distribution show that the differences are highly significant.

(2) *Egg samples:* These data include the frequencies of inversions observed in the offspring of single females inseminated in nature and placed in individual vials with the usual Drosophila culture medium. For statistical purposes the chromosomal structure was recorded in only a single larva from the progeny of each female. In the upper part of Table 2, the examined natural populations are indicated.

This type of analysis was extended to several stocks maintained for many generations under laboratory conditions. All these stocks were genetically very heterogeneous, each having originated from a mixture of the F_1 offspring of several females inseminated in nature. These were the same used to extract the data given in the upper part of Table 2. In the lower part of Table 2, the strains employed are indicated. The number after the name of the stock corresponds to the sequence of the analysis: Copiapó 1 and 2 were examined 21 and 33 months, respectively, after the establishment of the strain; Vallenar was analyzed 33 months after; Arrayan 1 and 2, 18 and 38 months after, respectively; Bellavista 1, 2 and 3, 32, 42 and 55 months, respectively; and Mendoza 29 months after the establishment of the stock. The typical egg-to-egg generation time in *D. pavani* is about 30 days. The stocks of Copiapó and Bellavista were the same used for the analysis of male samples.

The salivary gland chromosomes of *D. pavani* are not good enough to permit an easy identification of the gene arrangements in homozygous condition. For this reason, Table 2 shows only the data of the frequencies of both homozygotes and heterozygotes for the arrangements. In a random equilibrium condition the probability for a homozygote or a heterozygote for the gene orders in the left arm to be homozygous or heterozygous for the sequences in the right arm will depend entirely on the frequencies of the conditions in each arm. The expected proportions indicated in Table 2 have been obtained under such an assumption.

This Table 2 shows that, both in natural populations and in stocks maintained

TABLE 2

Observed (obs.) and expected (exp.) numbers of homozygous and heterozygous gene arrangements in the right (R) and left (L) arms of chromosome IV of Drosophila pavani

		Condition in the R and L arm of chromosome IV									
		Homozygous R				Heterozygous R					
		Homozygous L		Heterozygous L		Homozygous L		Heterozygous L			
Sample	Number	obs.	exp.	obs.	exp.	obs.	exp.	obs.	exp.	χ^2	Probability (D.f:3)
Natural populations											
Copiapo	62	21	17.0	12	15.9	11	14.9	18	14.0	4.08	...
Vallenar	48	19	14.7	6	10.4	9	13.4	14	9.5	6.66	0.09
Arrayan	114	28	27.8	18	18.1	41	41.1	27	26.8	0.03	...
Bellavista	159	54	41.9	17	29.0	40	52.0	48	35.9	15.24	<0.001
Mendoza	46	16	10.4	4	9.5	8	13.5	18	12.4	10.96	0.03
El Tabo	95	44	32.9	10	21.0	14	25.0	27	15.9	21.95	<0.001
Chillan	40	11	10.7	2	2.2	22	22.2	5	4.7	0.06	...
Viña del Mar	26	7	4.1	2	4.8	5	7.8	12	9.1	5.55	...
Olmue	22	6	5.0	4	5.0	5	6.0	7	6.0	0.73	...
Laboratory stocks											
Copiapo (1)	112	42	27.9	15	29.0	13	27.0	42	27.9	28.02	<0.001
Copiapo (2)	100	33	17.2	7	22.8	10	25.8	50	34.2	42.43	<0.001
Vallenar	100	27	10.2	7	23.8	3	19.8	63	46.2	59.89	<0.001
Arrayan (1)	399	149	151.3	49	46.6	156	153.6	45	47.3	0.30	...
Arrayan (2)	100	31	23.1	4	11.9	35	42.9	30	22.1	12.22	0.006
Bellavista (1)	417	120	97.6	74	96.3	90	112.2	133	110.7	19.17	<0.001
Bellavista (2)	100	32	20.6	12	23.3	15	26.3	41	29.6	20.87	<0.001
Bellavista (3)	200	96	55.6	12	52.3	7	47.3	85	44.6	131.40	<0.001
Mendoza	100	29	13.6	10	25.3	6	21.3	55	39.6	43.53	<0.001

in the laboratory, there is in most cases an overabundance of individuals which are either doubly homozygous or doubly heterozygous for their gene arrangements, apparently, as in the data in Table 1, a consequence of the non random association of the inversions. However, there is a difference between natural populations and laboratory strains. In the former the chi-square test for goodness-of-fit between observed and expected values was significant in only four out of nine samples. These results indicate that there are interpopulational variations respecting the association of inversions. In the laboratory stocks, on the other hand, the differences between the observed and the expected values are generally greater, and these differences are highly significant in eight out of nine cases. If the samples are compared, there appears to be a direct relationship between the number of generations which the strain has been kept in the laboratory and the degree of association between sequences. Thus, in the stock from Copiapó, 33 months after its establishment, the association of inversions was stronger than when the stock was 21 months old. Eighteen months after its establishment the stock from Arrayan shows no great differences from the expected chance association of its inversions; after 38 months in the laboratory, however, these differences were highly significant. The analyses of the stock from Bellavista 32, 42

and 55 months after its establishment show the same tendency towards an increase of the degree of association the longer the stocks have been kept in the laboratory.

DISCUSSION

The finding that the gene arrangements in the left and right arms of the fourth chromosome of *D. pavani* are non randomly associated is not unexpected in the light of results from other species of Drosophila and related diptera. As LEVITAN (1958) has pointed out, "two main causes, alone or in combination, could account for non random association: (1) absence of crossing over between the arrangements, and (2) natural selection acting against certain recombinant arrangements."

It is a well known fact that the effects of inversions on genetic recombination are not restricted only to the inverted section. Inversions in heterozygote form may also reduce the frequency of recombination along the rest of the chromosome (STURTEVANT and BEADLE 1936; DOBZHANSKY and EPLING 1948; CARSON 1953; LEVITAN 1954, 1955, 1958). Reduction of recombination by crossing over between linked inversions cannot explain their non random association in nature, however. According to the theoretical distribution of genes in a panmictic population, if any crossing over occurs at all, all possible combinations would be expected in proportions determined just by chance. Consequently, any observed non random association of inversions must be due to other causes, chief of which is probably the differential fitness of the recombination products.

In *D. pavani* for example, it seems clear that the main factor in the origin and maintenance of non random association of gene sequences is related to selective pressures. The distance between the complex of inversions in the left and right arms of the fourth chromosome covers more than 34 percent of its total length. All possible combinations between these arrangements have been observed in different frequencies in natural populations. In some places, such as Arrayan and Chillan, the observed number of the different chromosomal orders is just about that which could be expected due to chance. In other natural populations, however, as those of Bellavista and El Tabo, for example, the differences between the observed and the expected numbers are highly significant.

These observations suggest that in *D. pavani* the existence of a random equilibrium of the various alternative combinations of linked gene sequences, or the persistence of only some of them (the presence of both inversions in the same homologous chromosome, for instance) confer different adaptive value to the population. In each region, natural selection will favour the fittest condition.

The fact that the non random association occurs almost invariably in stocks maintained for a long time in the laboratory further emphasizes the primary selective nature of the phenomenon. Moreover, the older a culture, the greater the association of inversions in the same chromosome. This relation has been observed even in such a stock as Arrayan, which originated from a natural population in which the inversions were randomly distributed. The greater homogeneity of the results from laboratory stocks, in contrast to the heterogeneity

between natural populations, may be due to the fact that the former are subject to more intense and uniform selective pressures.

SUMMARY

The analysis of several natural populations and laboratory stocks of the neotropical species *Drosophila pavani* Brncic 1957, has shown that there is a non random association between gene arrangement complexes on the left and right arms of the fourth chromosome. The complexes are separated by over one third the chromosome's length. Non random association is observed in some natural populations and in all laboratory stocks examined.

There is a direct relationship between the number of generations a stock has been in the laboratory and the degree of association of the inversions. These data support the hypothesis that differential selection for chromosomes with certain combinations of linked arrangements is mainly responsible for this phenomenon. Apparently the existence of the two Standard arrangements or the two inversion arrangements together on the same chromosome is favored over chromosomes with Standard on one arm and an inversion arrangement on the other.

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GENETIC ANALYSIS OF A "PLATEAUED" POPULATION OF DROSOPHILA MELANOGASTER[1]

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SELECTION has long been recognized as a primary force in the evolution of species under either the natural or domesticated state. Yet perplexing cases occasionally arise where no response to artificial selection is observed in a population even though genetic variation is apparently present. This situation has been observed in *Drosophila melanogaster* populations under selection for such quantitative traits as bristle number (MATHER 1941; SCOSSIROLI 1954; RASMUSON 1955); wing length (ROBERTSON and REEVE 1952); and fecundity (BELL, MOORE and WARREN 1955). Similar results have been reported for poultry populations under selection for high egg production (LERNER and DEMPSTER 1951; DICKERSON 1955; and YAMADA, BOHREN and CRITTENDEN 1958).

Attempts to penetrate the haze surrounding the genetics of plateaued populations have led to such concepts as genetic inertia (DARLINGTON and MATHER 1949), coadapted gene complex (WALLACE 1953), genetic homeostasis (LERNER 1954) and genetic slippage (DICKERSON 1955). While differing in detail, these concepts present a common theme of genetic variation being conserved in a population at the expense of selection response. In some manner, genetic deviations beyond certain limits around the mean of a population place these individuals at a disadvantage from a fitness standpoint.

During a comparison of different methods of selection with *D. melanogaster*, a closed population under selection for high fecundity was observed to plateau early in the experiment. Marked inversions for all major chromosomes in this species provide a unique analytical tool to evaluate the importance of lethal, sterility and subvital factors in relation to the plateau for fecundity.

MATERIALS AND METHODS

The closed population of *D. melanogaster* analysed was the one developed in the second selection experiment reported by BELL, MOORE and WARREN (1955) after the population had been selected for high fecundity over a period of 47 generations. The population was reproduced each generation by selecting, on individual and family merit, 40 females along with their nonsib mates out of a total population of 400 pairs. One generation previous to the genetic analysis, the closed population was reduced to 20 families by combining the selected

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families two at a time. A rapid response to selection had been observed in this closed population during the early generations of selection; however, after reaching a peak of performance at the seventh generation no further progress was evidenced and the data suggested a decline. This decline occurred regardless of continued selection with the selection differential showing little change in magnitude.

The genetic analysis, which afforded an objective determination of the frequencies of deleterious factors in the closed population, was made possible by a marked-inversion technique (a modification of MULLER's method for constructing homozygous stocks) utilizing an analyser stock obtained from DR. A. B. BURDICK, Department of Biological Sciences, Purdue University and having the following genetic construction:

sc^{81} B In-S w^{a}sc^{8}; In-SMI, al Cy sp^{s}/Pm ds^{33k}; Ubx^{130} e^{s}/C Sb; pol.

The *B*, *Cy* and *Ubx* chromosomes contain the important recombination suppressors. Lines extracted by this technique are referred to as "isogenic" or "homozygous" even though the degree of homozygosity is not expected to be complete. The increased recombination between one pair of chromosomes due to the presence of a heterozygous inversion in a non-homologous pair was first pointed out by STURTEVANT (1919). However, our experience has led to confidence in the above marked inversions. Breakup of the marked chromosomes is rare and inbred lines formed by this method show a greater degree of inbreeding depression, an indirect measure of homozygosity, than that found in inbred lines formed by many generations of full sib-matings.

The type of genetic analysis employed in this study is unique with *D. melanogaster* and provided for the determination of sex-linked lethals plus autosomal sterility, subviable and lethal factors. The mating system used is shown in Figure 1. Virgin females from each of the 20 families, which constituted the closed population, were mated individually to males of the analyser stock as shown in Cross I. Approximately the same number of females was used from each family. The male progeny from Cross I would represent in their sex chromosome the gametic array of sex-linked genes from their dams, while the female progeny would have their sex chromosome from the closed population balanced with the marked sex chromosome from the analyser stock. Evidence of subviable or lethal sex-linked genes would be revealed by the sex ratio of progenies from the individual females in Cross I.

A single *Cy Ubx* F_1 male from the progeny of each Cross I mating was backcrossed to an analyser female in Cross II so that duplicate samples of chromosomes from the closed population would not be obtained. This male carried one wild type chromosome from each of the chromosome pairs in the original female. To duplicate in the Cross III progeny the same wild type chromosomes which were originally sampled, *B Cy Ubx* daughters of each Cross II were backcrossed to their respective sires. One hundred and thirty-two matings were made in Cross III and 126 reproduced.

If a recessive autosomal lethal were present in a chromosome sampled from the closed population, the homozygote in the Cross III progeny representing this

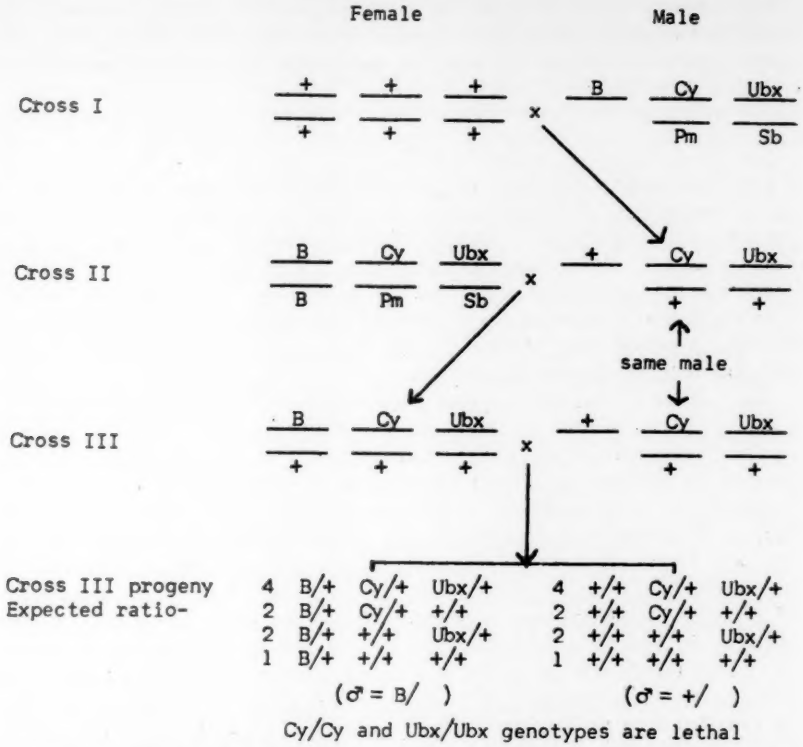
Female
Male
Cross I
Cross II
Cross III
same male
Cross III progeny
Expected ratio-
4 B/+ Cy/+ Ubx/+ 4 +/+ Cy/+ Ubx/+
2 B/+ Cy/+ +/+ 2 +/+ Cy/+ +/+
2 B/+ +/+ Ubx/+ 2 +/+ +/+ Ubx/+
1 B/+ +/+ +/+ 1 +/+ +/+ +/+
(♂ = B/) (♂ = +/)
Cy/Cy and Ubx/Ubx genotypes are lethal

FIGURE 1.—System of mating utilized for detecting subviable, lethal and sterility factors in the plateaued population (explanation in text).

chromosome would be missing. Thus, the frequency of lethals in a population and the chromosomes with which they are associated can be determined directly.

The ratio of fertile isogenic lines to the total directly measures the frequency of sterility factors in the closed population. Rather than a particular type failing to appear in the Cross III progeny, as with a lethal, it would instead be sterile.

The study was extended to differential viability among the progeny of Cross III when classified according to sex and chromosome type. By comparing through a factorial analysis the frequencies of the various types, the effects of heterozygosity, sex, and each of the major chromosomes on viability were observed. Also, differences in viability among the extracted wild type genomes were investigated.

In the plateaued population, genetic variation for the selected trait, fecundity, was estimated from data obtained on six of the "isogenic" lines and their hybrids. These estimates are in addition to those reported earlier on correlations among relatives (BELL, MOORE and WARREN 1957).

Experimental conditions including media, population densities, temperature, humidity and light were made as similar as possible to those provided for the closed population during the selection experiment and were thought to be optimum for fecundity.

RESULTS

Sex-linked lethals: The frequency of sex-linked lethals in the closed population was estimated from an analysis of the sex ratio of Cross I progenies resulting from the mating of virgin females from the closed population to analyser males. The progeny from 57 of the Cross I matings were counted and classified by sex. One count was made of the progeny two days after they began emerging. At this time a male was randomly chosen from each progeny group to be used in Cross II matings. A second count of these 57 Cross I progenies was made one week later and prior to the emergence of any F_2 flies.

Eight of the 57 samples analysed (or 14.04 percent) gave a significant deviation from a normal 1:1 sex ratio, with a shortage of males in every case. These results are summarized in Table 1. Of the eight which deviated from the normal, three of the samples (or 5.26 percent of the total) gave a good fit to a 2 female:1 male sex ratio which indicated the presence of a sex-linked lethal in the original female. When those samples which gave a significant deviation from a normal sex ratio were excluded from the analysis, the total sex ratio of the remaining 49 samples gave a significant deviation from a normal 1:1 sex ratio with an excess of females. This is evidence of subviability factors and suggested the relative viability analysis in a subsequent section. The observed frequency of possible sex-linked lethals was too low to be a major deterrent to selection response. In fact, the estimated frequency of sex-linked lethals is very little higher than would be expected from the reported natural mutation frequencies. DEMEREC (1937),

TABLE 1

Sex ratio analysis of eight Cross I progenies which gave a significant deviation from a 1:1 sex ratio and the goodness of fit of these to a 2 female:1 male sex ratio

Cross I mating	Number of progeny			Chi-square values	
	Male	Female	Total	1:1	2:1
1	105	166	271	13.73**	3.59
2	158	209	367	7.09**	15.45**
5	87	143	230	13.63**	3.37
6	77	107	184	4.89*	6.03*
95	66	91	157	3.98*	4.99*
157	86	130	216	8.96**	4.08*
172	58	88	146	6.16*	2.67
29	112	154	266	6.63**	9.18**
Remaining 49 matings	4060	4308	8368	7.34**	

* Significant at the .05 level of probability.
** Significant at the .01 level of probability.

in studies on mutability in *D. melanogaster*, reported the frequency of spontaneous lethals in chromosome I of various wild type stocks to range from 0.066 ± 0.03 percent to 1.09 ± 0.15 percent.

Autosomal lethals: Autosomal lethals could cause a population to respond no longer to selection if some mechanism were present which maintained the lethals at relatively high frequencies. In the event of lethal heterozygote superiority for the selected trait or for fitness, selection would tend to maintain the lethal and closely linked genes in equilibrium. Another mechanism might be a lethal located within an inversion with the inversion heterozygote superior to the homozygous wild type. Selection would favor the inversion, with a resulting increase in the frequency of the lethal.

Homozygotes for each chromosome of the genome originally sampled would be expected among the progeny resulting from Cross III of Figure 1 if no lethal factors were present. The failure to observe individuals homozygous for any particular chromosome pair would lead one to suspect a lethal factor in that particular chromosome.

Of the originally sampled genomes, 126 were successfully carried through the prescribed mating plan of Figure 1 and resulted in Cross III progenies which were recorded by sex and type. Individuals homozygous for all three major chromosomes were found in 113 (or 90 percent) of the progeny groups. If one considers that sex-linked lethal factors were eliminated at Cross I, then a liberal estimate for the frequency of autosomal lethal genomes is ten percent. One could accept this estimate and conclude that autosomal recessive lethal factors were not causing the plateau in selection response by either a balanced lethal complex or by superiority of the lethal heterozygote. However, there is evidence that the actual lethal frequency was considerably lower than 0.10.

Some of the Cross III progenies had relatively few individuals which made the presence of a complete homozygote unlikely since the probability of obtaining such an individual here was only one out of 18. This can be appreciated by observing in Table 2 the total number of individuals in each of the 13 Cross III progenies not yielding any homozygous individuals. With ten of the 13 cases having less than ten observations each, it is obvious that the estimate of lethals at ten percent is too high. To obtain a more realistic estimate, the remainder of Table 2 provides the probabilities of obtaining one or more of the complete homozygotes in each mating. If we assume no chromosomal interaction for lethality and a conservative 0.50 probability level, the frequency of genomes containing an autosomal lethal is estimated to be 7.1 percent. The frequency of second chromosome lethals was found to be 3.2 percent and that of third chromosome lethals was estimated to be 3.9 percent.

BERG (1941), in studies with the Nikita Botanical Garden populations of *D. melanogaster*, reported a lethal frequency of 14.49 percent among 1,905 chromosomes studied. WALLACE (1950) observed a lethal frequency of 4.6 percent after seven generations from an original lethal-free population. REEVE and ROBERTSON (1953) reported that lethal factors occurred in a population of *D. melanogaster* under selection for increased wing and thorax length which were associated with

TABLE 2

A summary of the 13 Cross III matings which could possibly possess autosomal lethal factors

Cross III mating	Homozygous chromosomes absent	Number of progeny	Probability of observing the absent genotype if nonlethal
59	II	9	.97*
152	II	58	.99*
204	II	48	.99*
215	II	17	.99*
285	III	2	.56*
294	III	1	.33
4	III	4	.80*
82	III	1	.33
371	III	3	.70*
375	III	3	.70*
53	III	6	.91*
58	II, III	2	.21
112	II, III	1	.11
Total number of autosomal lethals			9
Estimated frequency in the population based on the total sample of 126 genomes		9/126	.071

* Lethal assumed present for probabilities greater than .50.

the third chromosome. They concluded, however, that the lethal factors could hardly explain the refractory response to selection which they observed.

The observed frequency of autosomal lethals in the closed population under consideration in this study was as low or lower than that reported for nonselected populations. Obviously, lethal factors at a frequency of less than four percent did not contribute significantly to the lack of response to selection for high fecundity.

Sterility factors: Evidence suggesting that sterility genes were infrequent in the closed population is provided by its reproductive history. Each generation the population was reproduced from 40 single pair matings. Seldom did the frequency of unsuccessful matings exceed five percent.

The frequency of sex-linked sterility genes would be a direct function of the proportion of males in Cross II matings of Figure 1 which failed to produce progeny. These males would possess a random sex chromosome from the plateaued population while the autosomes would be heterozygous for the marked inversions. A total of 160 individual male matings were made in Cross II and 146 reproduced. If one assumed all 14 failures to be of genetic origin rather than environmental, the frequency of sex-linked sterility factors would be only nine percent. Autosomal recessive sterility factors would be made homozygous in the isogenic progeny by the outcross technique, and their effects would be observed by the inability of isogenic lines to reproduce. Less than five percent of the extracted lines were sterile or failed to reproduce in the first generation after extraction. Therefore, neither sex-linked nor autosomal sterility factors were

likely causes for the plateau in response to selection for fecundity in the closed population. In fact the frequency of sterility factors in this population is less than that reported by DOBZHANSKY (1951) for natural populations.

Differential viability

After eliminating the possibility of lethal and sterility factors as causes of the plateau in response to selection for fecundity, the possibility of recessive subviable genes was considered. These are usually associated with inbreeding depression. While deleterious when homozygous, they may display heterozygote superiority, as suggested by WALLACE (1950), due to overdominance or pseudo-overdominance. Such superiority of the heterozygote for the selected trait, fecundity, would cause a plateau in response even though genetic variation was present within the population. Likewise, heterozygote superiority for fitness would operate within the population to cause a plateau in response for the selected trait. By observing the relative viability of the various genotypic classes in the progeny of Cross III, it is possible to compare levels of homozygosity with inversion heterozygotes. In addition, these data made possible the identification of the genetic unit or factor with which the effect was associated.

Genetic factor analysis: The genetic factors of sex and the three major chromosomes, I, II, and III, were analysed in a 2^4 factorial manner. Sex was at two levels i.e., male or female, and each of the three chromosomes was at two levels i.e., homozygous wild type or heterozygous inversion type. The frequency of flies for each of the 16 classifications (2^4) in the Cross III progeny were grouped by the family from which the original females were taken and standardized to an equal expectation of 2.78 percent for the least frequent class (+/+ +/+ +/+) by the genotypic frequencies from Figure 1. The average values over all families are given in Table 3. For the factorial analysis, the data were transformed to arcsin $\sqrt{p}$, where p is the percentage of flies for each standardized classification

TABLE 3

Mean percentage of heterozygous inversion and wild type flies in Cross III progenies summarized by sex and genotype and standardized for expected genotypic ratio. Larger values indicate superior viability

Genotype by chromosome*			Relative viability**	
I	II	III	Males	Females
B/+	*Cy*/+	*Ubx*/+	2.28	2.80
B/+	*Cy*/+	+/+	2.71	2.67
B/+	+/+	*Ubx*/+	2.74	3.21
B/+	+/+	+/+	2.29	2.38
+/+	*Cy*/+	*Ubx*/+	3.21	3.03
+/+	*Cy*/+	+/+	3.18	2.40
+/+	+/+	*Ubx*/+	3.16	3.04
+/+	+/+	+/+	2.58	2.19

* Males are *B*/ or +/ for chromosome I.
** Expected value=2.78.

within each family. In three cases missing values were obtained by the method given by WALKER and LEV (1953). The transformed values were summed over all families, and the totals were compared in linear contrasts which were so made that differences, d, which were negative represent a superiority of females over males in comparisons involving sex, and homozygous wild types over the inversion heterozygotes in comparisons involving chromosomes. The variance of the transformed variable becomes that of the binomial distribution and takes the value of $\sum_{i=1}^{18} 821/N_i$, where N_i equals the total number of individuals produced in the ith family, and the fraction $821/N_i$ is summed over the 18 families which yielded data suitable for this analysis. With one degree of freedom the test gives an approximation to d/σ which is compared with the normal deviate.

When the totals for each sex over all Cross III progeny were compared in Table 4, no significant difference between the frequency of males and females was observed. This seems to contradict the significantly higher frequency of females among the progenies of Cross I (Table 1). Apparently the sex-linked low viability factors present in Cross I progenies were eliminated during the series of matings shown in Figure 1. Each wild type chromosome I was carried in the hemizygous male in Cross II matings, and through the outcross technique additional selection was exerted against sex-linked subvital genes by requiring these males to survive long enough to sire Cross III matings.

Further comparisons in Table 4 involve the homozygous wild type with the heterozygous inversion type for each chromosome pair. The homozygous wild type was superior at chromosome I, possibly of equal viability at chromosome II

TABLE 4

Genetic factor analyses involving the effects of sex, each major chromosome and their interactions on the relative viability of heterozygous inversion types versus *wild types in the Cross III progenies. The values approximate* d/σ *as described in the text*

	Genetic factors	Comparison values†
1.	Sex	0.02
2.	Chromosome I	−2.05*
3.	Chromosome II	1.30
4.	Chromosome III	3.90**
5.	Sex × I	−2.85**
6.	Sex × II	0.32
7.	Sex × III	−1.24
8.	I × II	−0.69
9.	I × III	−0.46
10.	II × III	−2.69**
11.	Sex × I × II	0.07
12.	Sex × I × III	0.62
13.	Sex × II × III	−0.36
14.	I × II × III	−1.01

* Significant at the .05 level of probability.
** Significant at the .01 level of probability.
† Males minus females where sex is involved; inversion type minus wild type where chromosomes are involved.

and inferior to the inversion heterozygote at chromosome III. Recessive subviable factors were rare in the closed population, if present. Although the marked-inversion chromosomes of the analyser stock are not normal, the vigor of the inversion heterozygotes in the Cross III progenies more nearly approaches that of wild populations than that of inbred populations.

The comparisons involving the main effects practically eliminated subvitals as a cause for the plateau in response to selection; however, the complete factorial was made and some of the interactions between the main factors are of interest from an evolutionary standpoint. The first three interactions, which involve sex with each of the major chromosomes, indicate whether or not the sexes differ in viability for any of the three major chromosomes over and above that due to the average effect of sex or the three major chromosomes alone. The sex of the individual influenced the relative superiority of chromosome I isolated from the closed population (Table 4). The values in Table 3 reveal two causes for this "Sex × I" interaction. The first is the superiority in chromosome I of the inversion heterozygous ($B/+$) female class over the hemizygous ($B/\ $) male class. The second cause is independent of the marked chromosome I and is interesting from an evolutionary viewpoint. The males exceed the females in the last four clases in Table 3. The two sexes in each class are summed over identical genotypes; however, the males are hemizygous for the wild type chromosome I while the females are homozygous for the same chromosome. These results provide evidence that sex-linked genes respond differently in the sexes when exposed to evolutionary forces. On the one hand, in females there would be selection for heterotic loci or coadapted gene complexes, as in most cross-fertilized diploid organisms. The reduced viability in the chromosome I homozygous females suggests this to be the case. On the other hand, the transmission each generation of a portion of the sex-linked genes through hemizygous males suggests strong selection for genes with favorable additive effects for viability. Superior viability cannot in this case be dependent upon heterotic loci. This dilemma is circumvented through a form of genetic dimorphism. Natural selection would favor those genes which produced superior viability when present either as a single dose in the hemizygous male or as diploid in females with superiority due to allelic interaction. Thus, one would expect inbreeding depression in homozygous females but not in males, with the consideration restricted to sex-linked genes. The results agree with this expectation.

Extending the analysis to the possibility of interaction between sex and chromosomes II and III, one finds in Table 4 that neither of these comparisons is significant. The common behavior of chromosomes II and III in both sexes would be expected from an evolutionary standpoint since the sexes presumably do not differ for these chromosomes.

Interactions between chromosomes, due to differential effects upon viability for the three major chromosomes, were tested by comparisons between heterozygous and homozygous types. These interactions, if significant, are indicative of epistasis between chromosomes for viability. The values for chromosomes "I × II" and chromosomes "I ×III" were not significant although the interaction of

chromosomes "II × III" was highly significant (Table 4). This reflects an epistatic effect between chromosomes II and III in favor of the homozygous wild type, and it is in addition to the average effect of each of the chromosomes alone. The absence of interaction between chromosome I and either of the other major chromosomes may be a function of the dual nature of selection on chromosome I. None of the three factor interactions are large enough to merit discussion.

Genome analysis: We have established that the wild type chromosomes extracted from the plateaued population possessed few lethal, subviable, or sterility factors. The viability of wild type homozygotes was about equal to that of the standard heterozygous inversion types. However, the factorial analysis does not measure genetic variation in viability among the wild type genomes.

The homozygous wild type for any particular chromosome sampled from the closed population cannot be compared directly with that from another genome since each set of Cross III progenies (see Figure 1) was reared in separate cultures. However, the viability of the homozygous wild type relative to the standard inversion heterozygote can be determined within each culture for each of the three major chromosomes and these relative viability values are comparable for all genomes sampled from the closed population.

The measured variable for viability becomes the ratio of flies which were homozygous wild type for each of the three major chromosome pairs and for sex to the total number of flies for the Cross III progeny of each genome sample, adjusted to an equal expectation according to the genotypic frequencies in Figure 1. The ratios were transformed to arcsin values for the analysis. Thus, each set of Cross III progeny, which represents a single wild type genome sampled from the closed population in Cross I, had six measurements reflecting the viability of each wild type chromosome when homozygous relative to the standard inversion heterozygotes present in the same culture. The six values by chromosome and sex represent I♂, I♀, II♂, II♀, III♂ and III♀. Cross III progenies with less than five individuals in any one classification were excluded. Such a restriction automatically excludes those few genomes previously identified as carrying lethal factors. Also, it might exclude some semilethals or subvitals; however, percentage values based on less than five individuals are unreliable. A total of 34 wild type genomes had Cross III progenies suitable for analysis. The variation in viability was statistically analysed for the three main effects of sex, chromosome, and genome, plus the interaction of these effects. The general model for the design is:

$$Y_{ijk} = \mu + S_i + C_j + SC_{ij} + G_k + SG_{ik} + CG_{jk} + SCG_{ijk},$$

where

Y_{ijk} is the arcsin $\sqrt{\text{percentage}}$ of wild type individuals of the total emerging in the kth genome for the jth chromosome in the ith sex.

μ is the average of all the Y_{ijk}.

S_i is the fixed effect of the ith sex; $i = 1,2$.

C_j is the fixed effect of the jth chromosome; $j = 1,2,3$.

SC_{ij} is the interaction of the ith sex with the jth chromosome.

G_k is the effect of the kth randomly chosen genome; $k = 1, \ldots, 34$.

SG_{ik} is the interaction of the ith sex with the kth genome.

CG_{jk} is the interaction of the jth chromosome with the kth genome.

SCG_{ijk} is the interaction of the ith sex with the jth chromosome with the kth genome and is used as the error estimate.

The analysis of variance for these viability data is presented in Table 5. The highly significant effect due to "Sexes" appears to contradict the comparison of "Sex" in the factorial analysis (Table 4). Actually, both analyses are reflecting the sex differences apparent in the summary of mean values given for genotypes in Table 3. In every case the hemizygous wild type chromosome I males (+/) are superior to the homozygous I females (+/+). This effect contributes to the significant mean square due to "Sexes" in Table 5, since this analysis was made on viability values for wild type chromosomes. The comparison in the factorial analysis of Table 4 included marked chromosome types as well as wild type. The superiority of $B/+$ females over $B/$ males has been pointed out earlier. Therefore, no over-all sex difference is observed over both marked and wild type chromosome I; however, these viability differences are revealed in Table 4 by the "Sex × Chromosome I" interaction. The highly significant effect shown in Table 5 among "Chromosomes" confirms other studies showing non-homologous chromosomes to vary in the magnitude of their effects on quantitative traits.

The most significant aspect of Table 5 is the small and insignificant variance due to differences among "Genomes" in contrast to the large and statistically significant "Sex × Genome" interaction. While not differing in their average effect on viability, the genomes exhibited differential behavior in the sexes. Apparently, those genomes contributing to superior viability in one sex lead to below average viability in the other sex. Presumably a type of genetic dimorphism had developed which would maintain genetic variation in a population. Those genes involved would reach an equilibrium frequency whereby positive natural selection within one sex would be counterbalanced by a negative selection in the opposite sex. In essence, this amounts to a negative genetic correlation between viability in the two sexes.

TABLE 5

Analysis of variation in relative viability among wild type genomes sampled from the closed population

Source of variation	Degrees of freedom	Mean square
Sexes (S)	1	192.64**
Chromosomes (C)	2	61.72**
Genomes (G)	33	6.48
S × C	2	11.24
S × G	33	24.46**
C × G	66	6.40
S × C × G	66	5.49

** Significant at the .01 level of probability.

Heterozygous versus *homozygous wild type:* The conclusion that the wild type genomes were not different in their average viability values rests on the comparison of homozygous wild types. While genetic variation in viability was present as revealed by the "Sex × Genome" interaction, another possible source in the original population and not revealed above would be a superior viability for heterozygous wild type individuals. Such a situation would not necessarily inhibit response to selection for high fecundity if genetic variation for the selected trait were present and not completely correlated with the viability factors. However, a comparison involving heterozygous wild types in addition to homozygous wild types was desirable as a further check on the type of genetic variation in the closed population plus a validation of genetic interactions found in the earlier analyses.

The homozygous lines extracted from the closed population had been reproduced by sib-matings for approximately 25 generations before the above analyses revealed the need for a comparison involving the heterozygous wild type. Because these lines may have changed during this period, new homozygous lines were extracted from each of three randomly chosen lines. This re-extraction of homozygous lines followed the same mating outline given in Figure 1. This provided the three "old" homozygous lines (184–1, 262–2, and 276–2), designated 1, 2, and 3, and their corresponding re-extracted "new" sublines 1a, 2a, and 3a. Virgin females from each of the six lines were mated separately with analyser stock males in the same manner as Cross I of Figure 1. The resulting *B*/+ *Cy*/+ *Ubx*/+ female progeny were then intercrossed with the +/ *Cy*/+*Ubx*/+ male progeny within lines and all possible combinations between lines as illustrated in Figure 2 to produce progenies which were classified by sex and genotype. They were expected to occur in frequencies identical to those for the Cross III progenies in Figure 1. The matings in Figure 2 are analogous to the combination of six inbred lines in all possible combinations; however, the hybrid progeny from reciprocal crosses were pooled to simplify the statistical analysis.

In order to contrast the viability of heterozygous wild types *versus* homozygous wild types, it is necessary to summarize these data in the same manner as described in the *Genome analysis* section. The measured variable was the ratio of flies which were wild type (homozygous or heterozygous depending upon the mating involved) for each of the three major chromosome pairs and sex to the total number of flies for each mating. The values were adjusted to an equal expectation according to the genotypic frequencies for Cross III progeny in Figure 1. The statistical model is the same as in the previous section except the main effect of "Genomes (G)" is more appropriately labeled "Matings (M)" since it represents the wild type genotype in either the heterozygous or homozygous state as prescribed by Figure 2. Each mating has six measurements, as before, which reflect the viability of either homozygous or heterozygous wild type chromosome pairs relative to the standard inversion heterozygotes in the same culture. In this manner a comparison is possible among wild type flies with different degrees of heterozygosity without the confounding influence of different culturing conditions. Obviously, such use of the standard inversion heterozygotes as environ-

mental controls makes the questionable assumption that genotype by environment interactions are negligible.

The analysis of variance for these viability data is presented in Table 6. The main effects "Sexes" and "Chromosomes" are highly significant as in Table 5.

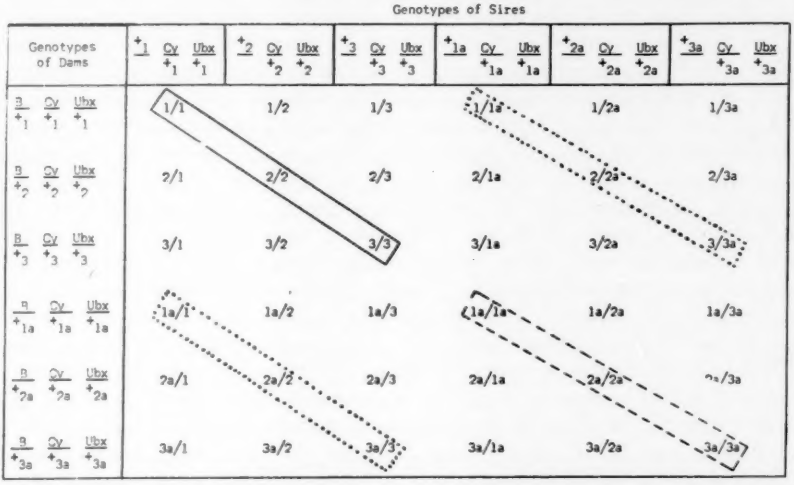

FIGURE 2.—Mating combinations involving marked inversion heterozygotes carrying wild type chromosomes from six homozygous lines. Listed in each cell are the sources of wild type chromosomes in the resulting progeny. See text for identification of those combinations which yield the different levels of heterozygosity.

TABLE 6

Analysis of variation in relative viability among the progenies resulting from the crossing of six "homozygous" lines

Source of variation	Degrees of freedom	Mean square
Sexes (S)	1	173.42**
Chromosomes (C)	2	343.49**
Matings (M)	20	10.22*
Between levels of heterozygosity†	3	4.41
Between homozygous "old" lines	2	1.69
Between homozygous "new" lines	2	46.50**
Between heterozygotes within lines	2	20.38*
Between heterozygotes between lines	11	4.91
S × C	2	8.90
S × M	20	16.31**
C × M	40	4.92
S × C × M	40	4.80

* Significant at the .05 level of probability.
** Significant at the .01 level of probability.
† Homozygous old lines *vs.* homozygous new lines *vs.* heterozygotes within lines *vs.* heterozygous between lines.

However, the most interesting aspect of Table 6 is the significant differences observed between matings. This is in contrast to the nonsignificant differences among the 34 genomes found earlier. These differences are due to (1) varying levels of heterozygosity, or (2) original genome differences not detected earlier, or (3) mutations within the homozygous lines since their extraction during the period of sib-matings. The design of the experiment diagrammed in Figure 2 makes possible a breakdown of the variation among the 21 matings to test these hypotheses. The data were partitioned according to homozygosity and heterozygosity of the wild-type chromosomes into four probable levels of heterozygosity. (1) Matings with all "+" chromosomes from the same "old" homozygous lines are enclosed by solid lines in Figure 2. (2) Matings with all "+" chromosomes from the same "new" lines are referred to as "homozygous new sublines" in Figure 2. (3) Matings with "+" chromosomes from an "old" line combined with those from its "new" subline are referred to as heterozygous within lines and these matings are enclosed by dotted lines in Figure 2. (4) Matings with "+" chromosomes from different lines, which include the remaining matings in Figure 2, are referred to as heterozygous between lines. This partitioning associates significant genetic effects with particular lines or crosses. Table 6 shows that the significant differences among "Matings" were not due to differences among levels of heterozygosity. Likewise the homozygous "old" lines, representatives of the original genomes, were not significantly different. Only the third hypothesis remains and the results are compatible with the occurrence of new genetic variation arising within the homozygous lines since their original extraction from the closed population. All significant differences among matings were limited to those involving the "new" homozygous lines.

The highly significant "Sex × Mating" interaction in Table 6 confirms the same type of interaction, "Sex × Genome", found earlier in Table 5 and supports the hypothesis of genetic dimorphism for viability. This sex by genotype interaction, also describable as a negative genetic correlation for viability in males and females, remains the sole identifiable source of genetic variation in the original plateaued population.

The selected trait: Not too many years ago one would have been consistent with population genetic theory to suspect that prolonged selection had fixed the "favorable" alleles and, thereby, eliminated genetic variation for the selected trait. Without harboring such an idea for the case at hand, the similarity in relative viability of the genomes from the plateaued population suggests that additional information is needed regarding genetic variation for the selected trait, fecundity.

Parent-offspring regression analyses of the fecundity data for each generation of selection in this closed population revealed that estimates of additive genetic variation were essentially zero for the generations showing no response to selection; yet, indications of nonadditive genetic variation remained (BELL *et al.* 1957). The marked-inversion technique makes possible an experimental test for genetic variability in the plateaued population by contrasting extracted genomes in both the homozygous and heterozygous state. Following the mating procedure

outlined in Figure 1, six new homozygous lines were extracted. These homozygous lines were crossed in all possible combinations including reciprocals. Daily egg numbers were obtained for two days (fifth and sixth days of age) on each of five daughters in each of three replications for each of the 36 crosses. An analysis of variation based on the cross means for each replication is summarized in Table 7. Highly significant differences observed among crosses indicate that genetic variation was present in the closed population even though it did not respond to selection. A breakdown of the 35 degrees of freedom for "Crosses" is especially enlightening. If the genetic variation were solely additive in nature, significance should be confined to the "Between inbreds" and "Between hybrids" effects, with the "Between levels" effect showing nonsignificance. But if the genetic variation were largely, or entirely, nonadditive, "Between levels" and "Between hybrids" should be significant, with "Between inbreds" being nonsignificant. The results support the hypothesis that the genetic variation in the closed population was nonadditive in regard to fecundity.

If the six inbred matings are excluded from these data, the remaining 30 F_1 and reciprocal crosses fit GRIFFING's (1958) Modified Diallel Design, Method 3. Provided the underlying assumptions are met, he has shown that this design yields unbiased estimates for the additive and nonadditive genetic variances in the parent population. The analysis of variance based on this model and presented in Table 8 reveals the general combining ability effects (GCA) to be nonsignificant with the corresponding estimated component of variance slightly negative. Since this component estimates one half the additive genetic variance in the parent population, we have another piece of evidence that the additive genetic variation for fecundity had been exhausted in this plateaued population.

An additional point of interest in Table 8 is the highly significant mean square associated with the specific combining ability effects (SCA). The corresponding variance component in the absence of epistatic effects provides an unbiased estimate of the nonadditive genetic variance in the parental population. This evidence confirms the earlier report (BELL *et al.* 1957) that genetic variation was present in the plateaued population under study, but it was nonadditive in nature.

TABLE 7

Analysis of variation in fecundity among the inbred and hybrid combinations of six genomes extracted from the closed population

Source of variation	Degrees of freedom	Mean square
Replications (R)	2	349.58
Crosses (C)	35	388.01**
Between levels of heterozygosity†	1	4962.54**
Between inbreds	5	163.90
Between hybrids	29	268.91*
R × C	70	142.17

* Significant at the .05 level of probability.
** Significant at the .01 level of probability.
† Inbreds *vs.* hybrids.

TABLE 8

Analyses of variance for the modified diallel crossing system involving F_1 and reciprocal crosses among six homozygous lines extracted from the closed population

Source of variation	Degrees of freedom	Mean square	Component of variance
Replications (R)	2	252.0	....
Crosses (C)	29	268.9	....
a. GCA	5	112.1	— 47.6
b. SCA	9	492.9**	164.8
c. Reciprocal	15	186.8	....
R × C	58	163.4	163.4

** Significant at the .01 level of probability.

DISCUSSION

The present genetic analysis of a "plateaued" population of *Drosophila melanogaster* has been more effective in revealing factors which have not contributed to the plateau rather than those responsible for it. This would constitute a serious criticism were not such factors as sterility, lethals, and subvitals widely reported to limit the response to selection. Selection experiments involving bristle number in Drosophila which illustrate this point are those of MATHER (1942), RASMUSON (1955), CLAYTON and ROBERTSON (1957), and SCOSSIROLI (1954). Other studies reporting such factors are those of ROBERTSON and REEVE (1952) with wing length in Drosophila and that by LERNER and DEMPSTER (1951) involving shank length in fowl. The distinguishing feature between the experiments discussed here and those reported in the literature is the fact that the selected trait, fecundity, is a major component of fitness. The significance of this point must remain largely a matter of conjecture until the selection response for fitness traits has been more thoroughly investigated.

The causes of a plateau must be scrutinized in light of their effects on the gain expected from selection. The expected gain ($\triangle \overline{G}_g$) in a trait per generation of mass selection may be expressed by the equation

$$\triangle \overline{G}_g = h^2 i$$

where h^2 is the heritability of the selected trait and i is the selection differential. Through either a reduction in effective heritability or by lowered selection intensity, several features may prevail which cause a decrease or plateau in response to selection (LERNER 1958). Obviously, a gradual worsening of the environment during the course of a selection experiment could lead to an apparent plateau when actually the average genetic merit would be improving. The performance of control populations and those under other methods of selection indicated that an environmental trend of this kind had not occurred in this experiment (BELL *et al.* 1955). Genotype-environment interactions in the presence of a fluctuating environment over generations could cause a plateau in response even though h^2 and i were positive within generations. Such a situation would be most difficult

to detect and would amount to an over-all reduction in the effective heritability. Likewise, heritability in a population could be reduced as selection exhausts the additive genetic variation. Increasing frequencies of lethal, subviable and sterility factors which are part of a superior heterozygote system will lead to an increase in the nonadditive genetic variance and eventually reduce the additive portion. An example of single gene heterosis associated with a lethal has been reported by MUKAI and BURDICK (1959).

The selection differential may decline due to a reduction in the phenotypic variation or due to a negative genetic correlation between the selected trait and reproductive fitness. In the extreme case where genes approach fixation from selection or inbreeding, the phenotypic variation would become reduced by the amount of the genetic variation. The selection intensity in turn becomes a function of the environmental variance and heritability approaches zero. A negative genetic correlation between the selected trait and fitness would reduce the effective selection intensity. The possible role of natural selection for some component of fitness to impede the effectiveness of artificial selection has not been eliminated in the present case. A more complete investigation should include other fitness traits, such as fertility and developmental rate and the relationship of these to fecundity.

Any thought that the closed population had reached a physiological or an environmental ceiling in fecundity can be discarded since other populations under different methods of selection consistently performed at higher levels even though they were initiated from the same base stocks and existed under quite similar environmental conditions.

After considering all available evidence, the major factor contributing to the lack of response in this particular plateaued population appears to be an exhaustion of the additive genetic variation due to prolonged selection. A significant amount of nonadditive genetic variation was observed and selection differentials remained large due to this nonadditive genetic variation plus environmental effects. The influence of lethals, semilethals, subvitals and sterility factors were found to be negligible. These findings agree with those reported by FALCONER and KING (1953) in their investigation of selection limits for body size in the mouse. A decomposition of the nonadditive genetic effects into those due to dominance, overdominance, epistasis, and genotype-environment interaction is limited until more refined statistical tools are developed.

SUMMARY

In an attempt to identify the genetic factors causing a plateau in response to selection for high fecundity in a closed population of *D. melanogaster*, 126 genomes were extracted from the population by the marked-inversion technique and the frequencies of lethal, sterility, and subvital factors were determined. The observed frequencies of these factors were sufficiently low to eliminate them as major causes for the lack of response.

Genetic variation for viability in the plateaued population as measured by

adult emergence was found to be associated with sex, the three major chromosomes, and a sex by genome interaction. While the extracted genomes did not differ in their average effect on viability, they exhibited differential action in males *versus* females. Such an interaction would be an effective force for retaining genetic variation in a population in spite of continued selection.

Evidence was presented which identified the exhaustion of the additive genetic variation in fecundity, the selected trait, as the primary cause for the plateau in selection response in this closed population. Nonadditive genetic variation was present to eliminate the possibility that selection, plus the inevitable inbreeding which occurs in a closed population, had rendered the population homozygous.

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THE GENETICS OF FLOWERING RESPONSE IN COTTON. III. FRUITING BEHAVIOR OF GOSSYPIUM HIRSUTUM RACE LATIFOLIUM IN A CROSS WITH A VARIETY OF CULTIVATED AMERICAN UPLAND COTTON[1]

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AS a result of cotton collecting expeditions in southern Mexico and Central America by J. O. WARE, T. R. RICHMOND, C. W. MANNING, and S. G. STEPHENS during 1946–48, some 600 introductions were brought to the United States. In the past two years, STEPHENS made additional collections in Central America and the West Indies; but only a few of these cottons have been studied in new plantings. Parts of the earlier collections have been grown at College Station, Texas; Santa Rita, Puerto Rico; Shambat, Sudan; Namulonge, Uganda; and Iguala, Mexico. On the basis of the African plantings and other studies, HUTCHINSON (1951) described seven taxonomic races of *Gossypium hirsutum* L.

In the tropical locations, the *G. hirsutum* introductions flowered the first season they were grown; but many of them failed to initiate fruit forms before frost in the latitude of the Cotton Belt of the United States. Almost without exception, the stocks which flowered in the Cotton Belt belonged to the race which HUTCHINSON described as *latifolium*. This race was considered by HUTCHINSON (1951) to be an annual form while the other six races—*morrilli*, *richmondi*, *palmeri*, *punctatum*, *yucatanense*, and *marie-galante*—were classified as perennials.

A. LANG (unpublished) found that types of cotton which failed to flower in the field at College Station, Texas, could be induced to flower by subjecting them to photoperiods of nine to ten hours; therefore, he considered them to be short-day plants. He was also able to induce flowering of the short-day types during long days by grafting them on a two-branched, day-neutral stock and then removing terminal buds, juvenile leaves, and fruit forms from the day-neutral portion after the branch had attained a length of 14 to 16 inches. At the same time, mature leaves were removed from the grafted branch.

A series of experiments was conducted to determine the inheritance of fruiting response in cotton. In the first paper LEWIS and RICHMOND (1957) reported the results of studies involving *marie-galante* and a day-neutral variety. In the second paper LEWIS and RICHMOND (1960) worked with crosses between short-

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day and day-neutral *G. barbadense* L. stocks. The present paper combines the results of two separate experiments, both involving short-day forms of race *latifolium* in crosses with a day-neutral upland, also of race *latifolium*.

Since KLEBS (1918) recognized the influence of light on plants, voluminous literature has accumulated on the effects of duration and quality of light on many developmental functions of plants. Comprehensive review articles have been written by WHYTE (1946), MURNEEK and WHYTE *et al.* (1948), WIGGLESWORTH *et al.* (1948), LEOPOLD (1951) and LANG (1952). The genetics of photoperiodism has not been investigated extensively, but enough reports are in the literature to show that the response of plants to length of day may have a monofactorial or a complicated multiple-factor genetic basis.

In an earlier paper of this series, LEWIS and RICHMOND (1957) reported on the inheritance of flowering response in a cross between short-day *G. hirsutum* race *marie-galante* and a self-pollinated line of an old agricultural variety of *G. hirsutum* (Deltapine 14). *Marie-galante* remained vegetative when grown in the field during the long days of summer but set fruit when grown during short days of winter in the greenhouse. Deltapine 14 set fruit in about the same length of time in both environments. Under long-day conditions all F_1 plants initiated fruit forms, but only about half the plants developed one or more flowers. In each backcross the recurrent parent was dominant; i.e., all plants of the backcross to *marie-galante* failed to flower under field conditions and all plants of the backcross to Deltapine 14 flowered. The segregating F_2 population did not reveal a simple genetic ratio; neither was the segregation typical of quantitative inheritance. Under short-day conditions, all plants flowered; but even with a favorable photoperiod *marie-galante* carried a lateness factor not associated with response to length of day.

In the second experiment, LEWIS and RICHMOND (1960) studied the inheritance of flowering response in a cross between Lengupa and Pima S-1, short-day and day-neutral stocks, respectively, of *G. barbadense* cotton. Under long-day conditions of summer, flowering was controlled by one gene pair and the short-day, nonflowering response was dominant to flowering. The monofactorial genetic basis of flowering response of this particular *G. barbadense* experiment was in sharp contrast to the complicated situation in the *marie-galante* experiment.

MATERIALS AND METHODS

The short-day parental material in these two studies was designated MW-84 (PI 163683) and MW-44 (PI 163643). Both of these stocks were collected by MANNING and WARE in Guatemala in 1948. The day-neutral parent in both experiments was an inbred line of Deltapine 14. According to HUTCHINSON (1951), all three stocks belong to race *latifolium*.

The basic plan of both experiments was essentially the same. The short-day parents were crossed with the day-neutral parent in the greenhouse during the winter, when both types would flower. Appropriate crosses and self-pollinations were made to produce F_2 and backcross seeds. The parental, F_1, F_2, and both

backcross populations were grown in the field during the summer of 1951 under long-day conditions (13–14 hours of light) and a similar planting in the greenhouse during the winter under short-day conditions (9–10 hours of light). A graph of the hours of possible sunshine at College Station, Texas, was published by LEWIS and RICHMOND (1957). In 1952, the field plantings of parental, F_1, F_2, and backcross populations were repeated and progeny tests made of selected segregants from the previous F_2 and backcross generations.

Field-grown plants were started in 6-ounce paper cups in the greenhouse and transplanted to the field at approximately two weeks of age, with 24-inch spacing within the rows and 40 inches between rows. A randomized block design with four replications was used in the field. In the greenhouse, potted plants were randomized on the benches and each progeny was replicated twice. The number of days from planting to the first visible fruit form (square), number of days to anthesis of first flower, and node number of first fruiting branch were recorded. In counting the nodes to the first fruiting branch, the cotyledonary nodes were recorded as nodes 1 and 2.

The parental stocks also were grown in the greenhouse in a curtained area where the exposure to sunlight could be controlled. One planting was exposed to a 9½-hour photoperiod during the winter and another to a ten-hour photoperiod during the summer.

One season MW-84 was grown at College Station and Lubbock, Texas; Sacaton, Arizona; and Brawley and Shafter, California, to determine the influence of environment at these locations on flowering.

The recording of the first square, first flower, and node of first fruiting branch on two separate experiments in both field and greenhouse resulted in a large amount of data. The results of the experiments showed that the same interpretation of inheritance could be made from either the squaring or the flowering data and that crosses involving MW-84 behaved essentially the same as those with MW-44. In the interest of economy of space, these data were condensed and in some cases not presented in tabular form if they were not pertinent to the interpretation of the results.

RESULTS

The means of number of days from planting to first square and to anthesis of first flower for MW-84 and Deltapine 14 under winter and summer greenhouse conditions are given in Table 1. Similar tests were conducted with MW-44, but they differ from the MW-84 data only in minor detail and are not presented in tabular form. The short-day cottons, MW-84 and MW-44, in the naturally short days of winter initiated fruit forms and developed flowers in about the same length of time as day-neutral Deltapine 14. In the longer days of summer, the short-day cottons failed to initiate fruit forms while Deltapine 14 flowered profusely under both long- and short-day conditions. By artificially shortening the photoperiod to ten hours, the short-day cottons were induced to square and to flower sporadically during the summer; however, the initiation and development

TABLE 1

Mean number of days from planting to first square and to first flower of a short-day and day-neutral cotton in four greenhouse environments

Season and day length	Population	Number of plants	Mean no. of days to 1st square	Mean no. of days to 1st flower
Winter	MW-84	6	45.0	80.8
(9½-hour day length)	D & PL 14	6	45.0	82.5
Winter	MW-84	15	41.7	66.7
(Natural day length)	D & PL 14	15	43.0	68.3
Summer	MW-84	10	No square	No flower
(Natural day length)	D & PL 14	10	43.5	62.5
Summer	MW-84	10	91.0	105.0*
(10-hour day length)	D & PL 14	10	50.5	76.0

* Mean of four plants; six failed to flower.

of flowers were markedly delayed. During the summer even Deltapine 14 developed more slowly under the light-control curtain than on the greenhouse bench.

Experiments with other crops have shown that temperature may modify photoperiodic response. In the present experiments, the greenhouses could not be artificially cooled; but thermograph records of the actual temperature were kept. Mean maximum and minimum temperatures during the summer were higher, on the order of 10° to 15°F, than the comparable winter temperatures. This temperature difference could account for the delayed initiation and development of fruit forms even under a ten-hour photoperiod; but whatever the cause, Deltapine 14 was less sensitive than MW-84 and MW-44.

The short-day MW-84 was grown at College Station and Lubbock, Texas; Sacaton, Arizona; and Brawley and Shafter, California, in 1953. None of the MW84 plants produced flowers at College Station, but all flowered at Shafter, California. The day-length was slightly longer at Shafter, but temperature records showed that Shafter had cooler temperatures. Flowering at Lubbock, Sacaton, and Brawley was late and sporadic.

The summary of data on the squaring and flowering behavior of parental, F_1, F_2, and backcross progenies involving short-day and day-neutral cottons is given in Table 2. Under the short-day conditions in the winter greenhouse all plants initiated fruit forms and developed flowers. Under these conditions MW-84 initiated and developed fruit slightly earlier than Deltapine 14; however, MW-44 was about seven days later. The mean number of days from planting to first square and first bloom in the F_1, F_2, and backcross to the short-day parent varied; but inspection of the data in Table 2 shows that there was never more than seven days difference in the means. Although this is significant statistically, the essential fact is that all plants in the short-day photoperiod of winter developed squares and flowers in approximately the same length of time. The backcross to Deltapine 14 in both cases was just as early as the parent, Deltapine 14, stock.

Under long-day summer conditions in the field, the squaring and flowering behavior of parental, F_1, F_2, and backcross population were strikingly different. Contrary to expectations, some of the short-day parental plants occasionally squared and sporadically developed a flower. This small amount of fruiting activity occurred relatively late, and in vegetative appearance these plants were similar to the nonflowering types. The day-neutral Deltapine 14 initiated and developed flowers in about the same length of time in the field as in the greenhouse.

The F_1 plants in the field flowered only slightly later than the day-neutral parent indicating that flowering response was partially dominant to nonflowering.

The F_2 and backcross to the short-day parent segregated into squaring and nonsquaring and flowering and nonflowering classes. Since the segregations did not reveal a classic Mendelian ratio, the data on the various progenies are presented as percentage of plants that squared or flowered. Actually, the ratio of plants which flowered to those which did not, particularly in the MW-84 study, strongly suggests a monofactorial basis of inheritance; but squaring data and progeny tests in the next generation do not support this interpretation.

The fact that a higher percentage of plants squared than flowered indicates that many plants in these populations initiated fruit forms which aborted. Of course, drought, insects, and other environmental factors cause squares to shed;

TABLE 2

Summary of data on squaring and flowering behavior of cotton grown during long days of summer in the field in 1951 and during short days of winter in the greenhouse at College Station, Texas, 1951–52

Progenies involving:	No. of plants under observation		Percentage of field* plants with		Days to first square†		Days to first flower†	
	Field	G.H.	Squares	Flowers	Field	G.H.	Field	G.H.
MW-84 and D & PL 14								
MW-84	45	5	4	0	84.5	49.0	. . .	79.0
D & PL 14	60	6	100	100	55.1±.4	53.3	76.3±.6	83.3
F_1 D & PL 14 × MW-84	58	6	100	100	60.1±.7	52.5	86.5±.4	84.2
F_2 D & PL 14 × MW-84	571	29	94	88	62.7±.5	50.2	84.4±.4	81.7
First backcross, F_1 × MW-84	345	18	66	50	67.4±.9	50.6	90.3±.9	83.1
First backcross, F_1 × D & PL 14	354	17	100	100	56.3±.6	50.0	78.4±.2	81.8
MW-44 and D & PL 14								
MW-44	38	6	71	3	83.1±.8	57.7	97.0	88.0
D & PL 14	43	6	100	100	56.6±.4	50.7	78.0±.4	83.1
F_1 D & PL 14 × MW-44	45	6	100	100	57.6±.5	51.3	80.8±.6	81.1
F_2 D & PL 14 × MW-44	419	30	99	90	60.7±.4	54.4	83.2±.4	85.3
First backcross, F_1 × MW-44	251	18	94	55	71.8±.6	54.4	93.0±.6	85.7
First backcross, F_1 × D & PL 14	238	18	100	100	55.7±.2	48.8	78.0±.2	78.6

* All greenhouse plants squared and flowered.
† Mean of those plants which squared and flowered.

but in these experiments, irrigation and insect control reduced these causes of shedding to a minimum. Facultative shedding appeared to be the major cause of some plants failing to flower after having initiated squares.

The backcrosses to day-neutral Deltapine 14 flowered in essentially the same length of time as Deltapine 14.

The following season the parental, F_1, F_2, and backcross populations were grown again with smaller populations, and progeny tests were run on segregants from the F_2 and backcross populations from the preceding year. Segregants were selected from the frequency distribution of flowering in order to progeny-test early flowering and progressively later flowering plants. Plants which squared but failed to flower and plants which failed to initiate fruit forms were also chosen. Nonflowering and late-flowering plants were moved to the greenhouse where seeds for the progeny tests were produced.

The flowering data of the 1952 study involving MW-84 and Deltapine 14 are recorded in Table 3. Data on squaring were recorded in a similar way; but since

TABLE 3

Frequency distribution of number of days from date of planting to anthesis of first flower of MW-84 × D & PL 14 in the field, 1952, College Station, Texas

Progeny	No. of days to first flower 1951	Flowering period in days										Number of plants that flowered	Mean no. of days to first flower*	Number of plants that failed to flower
		70	75	80	85	90	95	100	105	110	115			
MW-84	..	..	..	..	6	5	4	2	2	..	..	19	92.1	30
D & PL 14	..	..	9	11	..	..	..	..	..	..	..	20	77.7	..
F_1	..	..	..	15	5	1	2	..	..	..	..	23	82.8	..
F_2	..	1	14	23	9	6	6	1	1	..	..	61	82.6	8
F_1 × MW-84	..	..	..	2	9	2	3	1	..	..	..	17	87.7	4
F_1 × D & PL 14	..	..	22	20	1	..	..	..	..	..	..	43	77.3	..
F_3	75	..	20	31	16	4	..	..	..	..	..	71	80.3	..
	80	..	16	36	4	..	..	..	..	..	..	56	78.9	..
	85	..	14	35	8	3	1	..	..	..	..	61	80.4	1
	90	..	2	21	18	7	3	..	..	..	..	51	83.9	2
	95	..	8	23	13	8	3	3	1	..	..	59	83.8	5
	100	..	18	15	4	..	1	..	..	..	..	38	78.5	..
	105	..	1	10	4	3	..	..	..	..	..	18	82.7	..
	130	..	3	13	6	3	..	..	..	..	..	25	81.7	..
	145	..	..	3	8	4	2	..	1	1	..	19	89.1	1
	Sq. no flower	..	5	14	14	22	15	9	..	..	..	79	87.1	19
	No square	..	..	17	21	16	8	2	3	..	..	67	88.7	28
F_2 of (F_1 × MW-84)	75	..	8	19	3	2	..	..	..	..	..	32	79.3	..
	80	..	2	6	6	4	..	..	..	..	..	18	83.2	..
	85	..	9	21	9	3	1	..	..	..	..	43	80.9	3
	No square	..	5	19	25	21	12	5	1	1	..	89	81.7	22
F_2 of (F_1 × D & PL 14)	75	..	1	59	54	9	1	..	..	..	..	124	78.6	..
	80	..	..	90	129	21	3	..	..	..	..	214	78.0	..
	85	..	..	27	29	7	1	..	..	..	..	64	78.4	..
	90	..	..	8	9	6	1	..	..	..	..	24	79.6	..

* Mean of plants that flowered.

the same interpretation of inheritance is gained from both flowering and squaring data, the squaring data are not presented in tabular form. The correlation coefficient between flowering F_2 plants and the mean flowering data of F_3 progenies was .56 for the progenies with MW-84 as short-day parent and .81 for F_3 progenies involving MW-44. Both are highly significant. Of necessity nonflowering F_2 segregants could not enter into the calculations, and nonflowering segregants in the F_3 did not enter into the calculation of the mean. Thus the correlation coefficients were probably such that heritability of flowering response actually was higher than that estimated by the correlation coefficient.

The flowering data for the MW-44 study for both seasons are combined in Table 4. Examination of the data shows that more of the MW-44 parent plants flowered in 1952 than in 1951. The proportion of flowering to nonflowering plants was much higher in segregating populations in 1952. Eighteen flowering F_2 plants and seven nonflowering F_2 plants were progeny-tested in F_3. Although both the flowering and nonflowering F_2 plants segregated in F_3, the F_3 from the flowering F_2 plants produced a higher percentage of flowering segregants. These were an

TABLE 4

Segregation and mean number of days to flowering of various progenies of cotton grown under field (long-day conditions) at College Station, Texas

Progeny and year	Number of plants		Mean of plants* that did flower	Remarks
	Flowering	Nonflowering		
MW-44:				
1951	1	37	97.00	
1952	3	19	97.00±1.00	
D & PL 14:				
1951	43	0	78.05±0.40	
1952	24	0	77.33±0.38	
F_1:				
1951	45	0	80.76±0.60	
1952	26	0	79.27±0.37	
F_2:				
1951	379	40	83.20±0.37	
1952	77	1	82.05±0.76	
F_1 × MW-44:				
1951	139	112	92.96±0.59	
1952	40	8	90.03±0.84	
F_1 × D & PL 14:				
1951	238	0	78.00±0.21	
1952	46	0	78.00±0.48	
F_3: 1952	396	20	81.54†	From flowering F_2's
F_3: 1952	124	34	89.17	From nonflowering F_2's
F_2 of (F_1 × MW-44): 1952	113	7	85.70	From flowering F_1 × MW-44
F_2 of (F_1 × MW-44): 1952	58	49	89.45	From nonflowering F_1 × MW-44
F_2 of (F_1 × D & PL 14): 1952	568	1	78.54	

* Number of days from planting to first flower.
† Correlation between flowering dates of F_2 plants and F_3 progenies = 0.81**

average of eight days earlier than comparable F_3 segregants from nonflowering F_2 plants. With one exception, all plants from the F_2 of the backcross to Deltapine 14 flowered, and these progenies were essentially as early as Deltapine 14. The F_2 of the backcross to MW-44 segregated into flowering and nonflowering types, and the mean flowering date of those plants which did flower was later than that of plants from the backcross to Deltapine 14. This is true of the means, but individual plants were recovered in the F_3 and the F_2 of the backcross to MW-44 which flowered as early as Deltapine 14. The mean flowering dates for all generations for the two years were remarkably close.

The node number of the first fruiting branch and productiveness of the plants in the MW-44 study were recorded from a field planting and from a winter greenhouse planting. The data are presented in Table 5. The node number data reveal about the same pattern of inheritance as square and flower data. Under short-day conditions in the greenhouse, all plants developed fruiting branches, and the mean position of the first fruiting branch was very nearly the same in the parents, F_1, F_2, and backcrosses. In the field, under long-day conditions, it was observed that late-squaring and flowering plants were those in which squares and flowers occurred at a higher node on the main stem. The data on production, expressed as the mean number of bolls per plant, emphasize how barren the short-day plants were under long-day conditions. The backcross to Deltapine 14 by this measure proved to be as fruitful as Deltapine and about three times as productive as the backcross to MW-44.

TABLE 5

Summary of data of node number of first fruiting branch for six progenies of cotton grown under long-day and short-day conditions

Progeny	Node number of first fruiting branch												Number of fruiting branch	Total plants	Mean node of first fruiting branch	Mean number of bolls per plant
	6	7	8	9	10	11	12	13	14	15	16	17				
1952 field planting (long-day conditions):																
MW-44-5(#2+15)							1	2	2	4	2	3	25	39	14.9*	0.2
D & PL 14		5	14										0	19	7.7	18.7
F_1				15	8	1							0	24	9.4	15.4
F_2	1	3	19	26	12	5	5	2	0	2	1		1	77	9.5*	14.6
$F_1 \times$ MW-44				2	8	6	9	6	2	3	1	3	4	44	12.2*	5.7
$F_1 \times D \times$ PL 14		1	21	17	3	1							0	43	8.5	18.9
1952–53 greenhouse (short-day conditions):																
MW-44-5(#15)					3	8	1						0	12	10.8	...
D & PL 14				9	3								0	12	9.3	...
F_1			1	5	5	1							0	12	9.5	...
F_2				2	2	7	1						0	12	10.6	...
$F_1 \times$ MW-44					7	4	1						0	12	10.5	...
$F_1 \times$ D & PL 14				9	2	1							0	12	9.3	...

* Mean value of those plants in the progeny which had a fruiting branch.

DISCUSSION

The results of this study demonstrated that MW-84 and MW-44 plants squared and flowered sparingly or not at all during the long-day photoperiod of summer; but an environment was found in the naturally short days of winter in the greenhouse in which flowering occurred as soon as in the day-neutral parent. In the first paper of this series, LEWIS and RICHMOND (1957) found the *G. hirsutum* race *marie-galante* was inherently later than Deltapine 14 regardless of the environment in which it was tested. With the short-day *latifolium* the difference in time of flowering in comparison with a day-neutral stock can be eliminated by subjecting the plants to 10 to 12-hour photoperiods and moderate temperatures.

The lines of evidence obtained indicate that relatively cool temperatures may promote flowering of certain of the short-day cottons. (1) The plants exposed to ten hours of light during the summer in the greenhouse commonly were subjected to monthly mean maximum temperatures in excess of 100°F. Although flowering occurred, it was delayed in comparison to similar plants grown in the winter greenhouse where mean maximum temperatures were around 85°. (2) Flowering was promoted in the 1952 season as compared to 1951, and weather records show that 1952 was the cooler season. (3) Flowering occurred at Shafter, California, in 1953, in comparison with total failure to flower at College Station, Texas; and Shafter had the longer day-length and a cooler growing season, particularly a lower minimum temperature.

The ratios of squaring and nonsquaring plants and flowering and nonflowering plants in the segregating generations do not justify a simple genetic model for the inheritance of flowering response. However, the higher parent-progeny correlations and behavior of the backcross populations indicate that the difference in flowering response of these short-day and day-neutral cottons has a genetic basis. Flowering of the two *latifolium* stocks was partially dominant. This is in contrast to the complete dominance of nonflowering of short-day *G. barbadense* reported by LEWIS and RICHMOND (1960).

HUTCHINSON (1959) developed the thesis that Gossypium is essentially a genus of perennial shrubs native to the tropics and subtropics. In the tropics the perennial *hirsutum* stocks flower in the latter part of the rainy season and mature fruit in the dry season. According to HUTCHINSON, the onset of fruiting is governed by three major causes: (1) In young plants fruiting branches are not formed until a certain number of nodes develop on the main stem. This critical node number differs among cottons and is a genetic characteristic of the stock. (2) Photoperiod is a primary factor in the control of fruiting, and the stocks do not initiate flowers until the day-length is below 12 hours regardless of how many nodes the plant has developed. (3) Flowers may be initiated prior to the end of the rainy season, but the young flower buds shed if excessive water is available. When the rains abate and a favorable water balance is established, the fruit forms develop. When cottons were introduced into the southern United States, only a small portion of the types with a weak short-day requirement survived.

In the past 150 years, selection pressure on the most adapted original introductions led to the development of the upland cottons which have the ability to set fruit on branches arising from low nodes on the main stem, are day-neutral in photoperiodic response, and continue fruit production in wet seasons.

Apparently, the fruiting response of the short-day *latifolium* stocks used in this study differs from that of cultivated day-neutral types largely by their reaction to photoperiod. Certain temperatures or other environmental effects may modify the response. This difference is conditioned by a complex multiple factor genetic mechanism. In *marie-galante* cottons, photoperiod is only one of the factors influencing flowering; seemingly the plants require a minimum number of nodes before flowering begins.

On the basis of one experiment by LEWIS and RICHMOND (1960), the difference in flowering response of short-day and day-neutral *G. barbadense* is controlled by one gene pair and has had an independent evolution from *hirsutum* in this respect.

Certain practical considerations are evident from these studies. The nonflowering habit of introduced cottons can be eliminated in segregating generations when crosses are made with day-neutral stocks. Thus, the short-day response does not, *per se*, eliminate introduced cottons as sources of breeding material. In the past, sensitivity to the long summer days of the temperate zone acted as a barrier to the establishment of many stocks of cotton which thrive in the tropical zone. In the tropics, all the stocks of *G. hirsutum* classified as race *latifolium* appear to have similar gross characteristics. However, certain of them may carry genes for valuable economic characters in the temperate zone. Methods for exploiting the potential variability that has been locked in the short-day plants of race *latifolium* may now be devised.

These experiments were conducted in the field and in the greenhouse under constantly changing photoperiods and under the various total environments that existed during the course of the experiments. Climate-control chambers were lacking, and doubtless research with such equipment will be necessary to establish critical photoperiods and to determine the interaction of light and temperature.

SUMMARY

Fruiting response in cotton was studied in crosses involving short-day and day-neutral stocks from the same taxonomic race, *G. hirsutum* race *latifolium*. The data lead to the following general conclusions:

(1) Stocks belonging to the same taxonomic race may differ in response to length of day.

(2) Exposure of the short-day *latifolium* stocks used in this experiment to an inductive photoperiod induced them to flower as soon as day-neutral stocks.

(3) The short-day and day-neutral responses were primarily under genetic control; the day-neutral reaction was partially dominant.

(4) The effects of short-day photoperiod can be modified by other factors, particularly temperature.

(5) Short-day cottons, which may contribute useful characteristics, can be utilized in applied breeding programs in the temperate zone.

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R-LOCUS EYE-COLOR FACTORS IN MORMONIELLA[1]

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IN the chalcidoid wasp *Mormoniella vitripennis* (Walker) there has been formed by mutation a series of multiple allelic genes, each differing from wild type in one or more factors. The term "factor" is here used in an inclusive sense to denote anything which has an effect on development or which can in any other way be detected. The genes in this "R" series are pleiotropic, unifactorial, bifactorial, trifactorial, etc., according to the number of their factors in which they differ from wild type. Any one factor may have one or more mutant states which are *alternative* to each other. The *alternative states* of the *factors* form subseries within the series of allelic *genes*, so that, theoretically at least, the total number of possible alleles would be equal to the product of the various states of each of the factors.

Factors O, S *and* M: Two of the eye-color factors are characterized by relatively high frequency of mutation, either spontaneous or radiation induced. Factor *O* mutates to colorless, oyster-white, and factor *S* mutates to scarlet. The compound wild-type females, *oy*.+/+.*st*, produce oyster and scarlet sons in equal numbers from their unfertilized eggs. The double recessive gene, *oy.st*, is formed not by recombination of the factors, but by mutation in stock already recessive for one factor.

Mutations comparable to oyster and scarlet have been obtained in a related chalcidoid species, *Pachycrepoideus dubius* Ashmead, belonging to the same family Pteromalidae as Mormoniella (WHITING 1954). Here also the two genes act as complementary alleles and the rate is high, five scarlet, six oyster among 1433 sons of X-rayed (2500r) females. It has been suggested that an *R* region may be common to the group. The *O* factor produces some enzyme which, acting alone on chromogen, produces red pigment while *S* determines the presence of a secondary enzyme which may unite with *O*. The compound enzyme *O–S* combines with chromogen to form dark pigment. The proximity of *O* and *S* in the *R* region may facilitate production of compound enzyme *O–S*, leading to chromatogenesis. Mutations to oyster and to scarlet are relatively stable.

Gene descendants of a certain mutated gene, *ti-277* (*ti.st*) (tinged eyes) are characterized by an unstable condition of factor *O* giving a range of "red" eye colors from reddish brown (*rdh*) near wild type, through mahogany (*mh*), dahlia (*da*), tomato (*to*), apricot (*ap*), orange (*or*), peach (*pe*) to tinged (*ti*) near oyster white. None of these relatively frequent spontaneous mutations is accom-

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panied by any deleterious condition such as inviability or sterility, which is often associated with radiation-induced changes. There have been induced in factor *O* and in factor *S* a very few apparently stable mutations giving eye colors intermediate between wild type and oyster in factor *O* and between wild type and scarlet in factor *S*.

A test for locating eye-color mutations with respect to *O* and *S* is to cross the mutant male to compound females *oy*.+/+.*st*. If the F_1 females are all wild type, neither *O* nor *S* is involved; if they are all mutant type, both *O* and *S* are affected; if half are mutant type, the mutation occurred in one of the factors and an F_2 generation will determine whether this is *O* or *S*.

SAUL (1957) presented evidence for the existence of a third factor, *M*, at the *R* locus. Two separate mutations, one X-ray-induced, *da*-845 (*mh*-5101,GBS), the other fast-neutron-induced, *da*-846 (*mh*-846,GBS), cause dahlia eyes, very similar or identical in appearance. F_1 females, *da*-845/*da*-846, resemble the parental stocks but crosses with *O*-factor or with *S*-factor recessives or with double, *O.S*-factor, recessives, *oy.st*.+ for example, give wild-type females, *oy.st*.+/+.+.*da*, which produce sons of the two parental types only.

Use of the same symbol, *da* for example, for states of different factors indicates similar dark red eye color but implies nothing respecting the chemical nature of the pigment. X-radiation of the two stocks, *da*-GF (*da*.+.+) and *da*-846 (+.+.*da*) produced *st*-426 (*da.st*.+) and *or*-806 (+.*st.da*), both *st* in *S* but with very different eye colors. It has been found that changes in *O*, unless they are extreme, have no appreciable effect on scarlet eye color. A comparable change to *da* in *M* appears to remove much of the bright red pigment reducing scarlet to orange.

Masking, epistatic, effects are shown by *oy* and *st*. With an *oy* state in *O* no eye pigment can develop regardless of other factors at *R* or at other loci, and with a *st* state in *S* no dark pigment can be formed.

CASPARI (1958), X-raying wild-type males and crossing them to *pe*-333.5 females, obtained many mutations to *oy* and to *st* and a few to dark red. One of these, *da*-817, associated with an egg lethal, proved to be in *S*, giving mutant-type compounds with *S*-factor genes, but wild-type compounds with *O* and *M*. A second, called *mh*-819, has wild-type eyes in males and in homozygous females. The dark red, mahogany, character appears distinctly in compounds with *O*-factor recessives, but not with *S* or *M*.

A third mutant type, *da*-838, has eyes indistinguishable from *da*-845 and *da*-846, and the compound females are likewise similar. There are, then, three *M*-factor mutations. However, *da*-838 differs from *da*-845 and *da*-846 in that compounds with *oy*-DR and with other recessives in *O* are distinctly reddish brown contrasting with the dark brown wild-type compounds with *st*-DR. The former may be expressed as *rdh*.+.*da*/*oy*.+.+, the latter as *rdh*.+.*da*/+.*st*.+. Gene 838 has a reddish, *rdh*, condition in factor *O* which is masked in the homozygote by *da* in *M* and in the compound with *st* by complementary allelism. This condition of *O* is differentiated by genes which, like *oy*-DR, are recessive (*oy,ti, pe*) in *O* and + in *M*.

Status of the *R*-locus eye-color factors, especially the unstable-*O*, as it appeared

a year ago, is presented in a recent paper (WHITING 1960). Now with accumulating evidence, a certain correction must be made and additional data are to be given.

NEW DATA

The nonexistence of factor N: A fourth *R*-locus factor, *N*, was postulated by CASPARI (1958) on the basis of a dark red mutation, lethal at eclosion, *da*-835, which "was found to have arisen from neither *S* nor *O*. It appeared first as a mahogany mutant when in compound with peach, but the sons of this individual segregated as peach and dahlia rather than peach and mahogany. Dahlia is lighter than mahogany, so it seems as though the peach stock is demonstrating something less than wild type in a fourth factor of *R*." The eye-color formula of mutant gene 835 was supposedly +.+.+.*da* and of *R*-locus peach, *pe.st.*+.*mh*. Partial complementarity would then give an eye color, mahogany, in the compound females darker than that of their haploid dahlia brothers dying at eclosion.

Tests were made to determine the presence of a factor-*N* change in several different *R*-locus mutant genes. Their compounds with *da*-835 were examined and in most cases compared with the compound *da*-835/*pe*-333.5. Five genes known to be mutant in *O* but not in *S* or *M*, the *M*-factor genes *da*-845 and *da*-846 (+.+.*da*), *da*-838 (*rdh.*+.*da*) and *oy*-825 (*oy.*+.*da*) (from X-rayed *da*-846 stock) all gave compounds which were definitely dark brown wild type.

By contrast, the compounds made with 13 genes mutant in *O* and *st* in *S*, with five genes + in *O* and *st* in *S*, with *or*-806 (+.*st.da*) (from X-rayed *da*-846 stock) and with *vm*-MK which is + in *O*, *vm* in *S*, showed mahogany eye color. Compounds with *mh*-605 (+.*mh.*+), and with *oy*-848 (*oy.mh.*+) (from X-rayed *mh*-605 stock) are very dark but usually separable from wild type. *Da*-835 acts like a dominant (or partial dominant) over *st* in the *S* series of alternatives. Eye facets of the haploid males dying at eclosion are well delimited, but eye color is obviously lighter than that of their *st*-compound sisters. This is probably in part due to the fact that they are males, and males are likely to have lighter eyes than females, presumably because they mature more rapidly giving less time for chromatogenesis. In the present instance also the recessive lethal *lx*, associated with gene 835, terminates life at eclosion and may well have some pre-eclosion effect.

A guess at the potential intensity of eye pigmentation permitted by gene 835 may be made by comparing the colors obtained in different combinations of *S* alternatives which for convenience may be designated *st*, 605, 835 and +. Females 605/*st* are markedly brighter than 835/*st* and are easily separated from them. They are roughly comparable to the inviable "dahlia" 835 males. 835/*st* females closely resemble "mahogany" 605 males which in turn are lighter than females 605/605. These vary from *rdh* brown to mahogany. Females 835/605 average still darker, fluctuating from wild type to reddish brown. Presumably then, 835 mature males and 835/835 females, could they be obtained, would be indistinguishable from wild type. The series arranged in order of increasing pigmentation would be 835 immature = 605/*st* < 835/*st* = 605 < 605/605 < 835/605 < (?)835 mature = (?)+ < (?)835/835 = (?)+/+. Gene 835 may be designated

mh-835 in agreement with the color of the *st*-compound females rather than with that of the immature males. At this point its formula may be expressed +.*mh*. +.*fsa.lx*, the first three symbols expressing states of factors *O*, *S* and *M* respectively while *fsa* and *lx* show homology with other female steriles and nonhomology with other lethals.

The fourth eye-color factor P: While data were being collected indicating that *mh*-835 is a very dark mutant state of factor *S* rather than a change in a fourth factor *N*, another mutant eye color was found in a factor *P*, which may take the place of *N* as the fourth *R*-locus eye-color factor. Dr. G. B. Saul obtained a female-sterile mutant type which may be called *da*-442, having eye color closely resembling *da*-845, *da*-846 and *da*-838. Its compound with *pe*-333.5 (*pe.st.*+.+) is very dark wild type indicating that the change is neither in *O* nor in *S*. Such compound females produced 574 dahlia and 685 peach males suggesting a somewhat reduced viability of the former. Daughters sired by peach males were 109 wild type and 110 peach and daughters sired by *da*-442 males were 119 wild type and 95 dahlia.

The compound females were crossed with *st*-689 males (+.*st*.+.+.*fsa*.+) and produced wild-type (+.+.+.*da*.+.*fsb*/+.*st*.+.+.*fsa*.+) and scarlet (*pe.st*.+.+.+.+/+.*st*.+.+.*fsa*.+) daughters and dahlia (+.+.+.*da*.+.*fsb*) and peach (*pe.st*.+.+.+.+) sons. From these, a *da*-442/*st*-689 stock was derived in which males were dahlia and scarlet, females wild type, dahlia and scarlet. The homozygous dahlia and scarlet females are near-sterile, the former homozygous *fsb*, the latter homozygous *fsa*. Eye color and fertility of the wild-type females are produced by complementary allelism.

The compound *da*-442/*pe*-333.5 females were also crossed to *oy*-423 males (*oy.st*.+.+.*fsa*.+) and produced daughters, wild type (+.+.+.*da*.+.*fsb*/*oy.st*.+.+.*fsa*.+), and tinged (*pe.st*.+.+.+.+/*oy.st*.+.+.*fsa*.+). *Oy*-423, like *st*-689, carries *fsa* so that it was possible here also to derive a balanced stock, *da*-442/*oy*-423, with males dahlia and oyster, females wild type, dahlia and oyster.

Homozygous *da*-442 females are not only near-sterile, but many of them have wrinkled wings and are unable to sting the host fly pupae or to feed. Of 83 such females in one test, only two were alive after six days in the incubator. Offspring numbered 26. In another test, 86 homozygous females produced no offspring. Only two host pupae were put in the vial. One of them showed four sting marks, the other, eight, indicating that one, at least, of the females was able to puncture the puparium. From 29 homozygous females set with four pupae in another test, ten progeny were obtained and from 38 in another there were 17. That it may be possible to increase fecundity of homozygous *fsb* females is indicated in a test in which 31 females with normal wings were selected and set with four pupae. Offspring totalled 117.

Sterility caused by *fsb* is of a very different type from *fsa* sterility. The latter, which is carried by mutant genes *st*-689, *st*-829 and *oy*-423 and is characteristic both of the homozygous and of the compound females, does not malform the sting. The females are able to penetrate the puparia, form coagulation tubes for feeding

and inactivate the pupae, but they rarely lay an egg. They are, therefore, ideal as "nurse-wasps" in certain experiments concerned with rearing offspring from counted eggs (RAY 1955).

Da-442 males were crossed with *mh*-835/*pe*-333.5 females. The daughters were all wild type and fertile, +.*mh*.+.+.*fsa*.+.*lx*/+.+.+.*da*.+.*fsb*.+ and *pe.st*.+.+.+.+.+/+.+.+.*da*.+.*fsb*.+. By crossing these daughters to *da*-442 males and selecting the progenies containing no peach males (and having the inviable dahlia pupae) a balanced stock was derived of fertile wild-type (835/442) and near-sterile dahlia (442/442) females, viable *da* 442 males and inviable *da* 835 pupae.

No difference could be noted when freshly eclosed males of *da*-442 and *da*-845 were compared, both being a rather uniform dark red without a fleck. When *da*-845 females were crossed with *da*-442 males, the daughters were very dark wild type. These, mating with their *da*-845 brothers, produced wild-type (442/845) and dahlia (845/845) daughters, the latter being fertile and without wrinkled wings. Some of the wild-type compound females were crossed to *da*-442 males. These produced wild-type (845/442) and dahlia (442/442) females, many of the latter having wrinkled wings. Sons of the compound females were dahlia as expected (442 and 845).

Wild-type compound females (835/442) were crossed with *or*-300.5 males (*or.st*.+.+.+.+). The wild-type daughters (442/300.5), having mated with their *da*-442 brothers, produced wild-type (300.5/442) and dahlia (442/442) daughters, the latter including many with wrinkled wings. Sons were dahlia and orange. The mahogany daughters (835/300.5) (from 835/442 × 300.5) produced viable *or*-300.5 and inviable *da*-835 sons. Their daughters, sired by their *da*-442 brothers, were all wild type, the two genotypes 835/442 and 300.5/442 being indistinguishable.

DISCUSSION

With respect to a complex series of alleles such as the *R* series in Mormoniella, it is important first to clarify definitions. The *R region* of the normal wild-type chromonema, the region in which the alleles are formed by mutation, consists of genetics materials and structures governing the course of normal development. In the *vicinity* of this region are other regions within which mutant genes may be formed showing linkage with the *R* genes. When a mutation occurs in the *R* region a *locus* is formed, a *point* in the genetics sense, acting as a unit in the meiosis of the heterozygote or of a compound. There is no reason to suppose that the *R* region in the homozygous wild type or in a homozygous mutant type acts as a unit in meiosis. The region may be very long so that much crossing over may occur, although undetected because of lack of markers. Also, any given mutation may not involve the entire region so that the genetics *point*, *locus*, has a different physical basis in one heterozygote (or compound) than it has in another.

The *R* region has no definite limits. It may be extended by one mutant gene much further than by another. Thus *st*-841 prevents crossing over with purple (*pu*) body color which "normally" is 11 units distant. If we had only *pu*, *st*-841

and *oy*-423 *pu*, we might call this a triple allelic series with *pu* as a recessive alternative state of factor *Pu*. Because "the purple gene" is wild type in its factors other than *Pu*, because *st*-841 is near-sterile and because *oy*-423 is female-sterile, the factors involved would be *O*, *S*, *A*, *Y*, *Pu*, and the gene formulae would be, respectively, +.+.+.+.*pu*, +.*st*.+.*ns*.+ and *oy.st.fsa*.+.*pu*. Purple, which has been taken in the wild several seasons at Woods Hole, Massachusetts, crosses over with all other *R*-locus genes obtained. *St*-841 always has wild-type, pu^+, body color and a dominant factor may be added to its formula, *CpuO*, preventing crossing over with purple. This factor may very well be one or more associated inversions. A gene, then, includes anything and everything in a given region which has been changed by a mutation and which segregates as a unit.

It seems likely that any region of the active chromonema might possess factors furnishing materials for mutation to produce a series of allelic genes. A visible mutation serving as a marker for the region would be necessary as a starting point in a project to obtain multiple alleles by which the possibilities might be explored. For this, the *R*-region had a great advantage in that there were already two factors, *O* and *S*, each highly mutable so that a doubly recessive gene, *pe*-333.5, was easily obtained. By X-raying wild-type males and crossing them to peach purple females numerous scarlet and oyster mutants were produced which differed in factor states modifying viability and in male and female fertility. In this manner also the much less frequent intermediate color mutants of *O* and *S* appeared and at least one double mutant, *da*-838(*rdh*.+.*da*.+), revealing a third factor, *M*, change as well as one in *O*.

The four *R*-locus eye-color factors, *O*, *S*, *M* and *P*, may very well be considered as cistrons (BENZER 1957). The spontaneous mutations occurring in the unstable-*O* pedigree (WHITING 1960) may be due to intracistronic recombination (NELSON 1959). This should be studied by use of marker genes to left and right of *R*. The many *R*-locus factors affecting viability and fertility constitute a very complicated structure in a restricted germ-plasm region.

SUMMARY

The multiple allelic series formed by mutation in a restricted chromonemal region, *R*, involves many different factors affecting viability, fertility and eye color. Each of these factors exists in two or more alternative conditions or states.

Factor *O* most frequently mutates to oyster white (eyes), very infrequently to intermediate "red" colors, except that in a certain pedigree, unstable-*O* spontaneous mutation is frequent.

Factor *S* most frequently mutates to scarlet (eyes), very infrequently to intermediate dark red colors.

Three mutations have been found in a third factor, *M*, causing dark red, dahlia, eyes.

A fourth factor, *N*, was postulated on the basis of a dark red mutation, but tests have now shown that this is in the *S* series giving a very dark mahogany in compound with scarlet.

Factor *P*, now replacing *N* as the fourth eye-color factor, is based on a dahlia gene, acting as a complementary allele to mutant eye colors in the other three series.

These four eye-color factors may be cistrons and the spontaneous mutations of factor *O* due to intracistronic recombination.

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CYTOGENETIC ANALYSIS OF THE ACTION OF CARCINOGENS AND TUMOUR INHIBITORS IN DROSOPHILA MELANOGASTER. X. THE NATURE OF THE MUTATIONS INDUCED BY THE MESYLOXY ESTERS IN RELATION TO MOLECULAR CROSS-LINKAGE

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IN efforts to establish a correlation between the chemical structure of "nitrogen mustards" and their tumour inhibitory activity, it was noted that the presence of two active groups per molecule was more favourable (HADDOW, KON and ROSS 1948). Seeking an explanation for this difunctional requirement, the cross-linkage hypothesis was postulated (GOLDACRE, LOVELESS and ROSS 1949). This suggested that two active groups might be required to permit the molecule to react at two distinct points lying either on a single fibre, or more especially on two contiguous fibres.

Mutagenesis studies did not support the cross-linkage hypothesis. The monofunctional compound ethylene oxide proved to be decisively mutagenic (BIRD 1952), though at the same molar dose it was still far less effective than the corresponding difunctional compound diepoxybutane (FAHMY and FAHMY 1956). More significant in this connection is the situation with the mesyloxy esters, where it was found that the monofunctional compounds (the methyl and ethyl derivatives) were highly active mutagens (FAHMY and FAHMY 1957). At the same molar dose, the above monosulphonates were considerably more mutagenic on mature sperm than the majority of the difunctional derivatives (FAHMY and FAHMY 1956 and unpublished).

Recent physicochemical studies on the *in vitro* reactions of alkylating agents on model macromolecules, as well as on deoxyribonucleic acid (DNA) have revived interest in cross-linkage (ALEXANDER, COUSENS and STACEY 1957; STACEY, COBB, COUSENS and ALEXANDER 1958). Evidence was available for the occurrence of inter- and intramolecular cross-linkage under difunctional compounds, which resulted in marked alterations of the physical characteristics of the DNA molecules (changes in molecular weight and viscosity). In contrast, it was possible to demonstrate that monofunctional compounds produce separate and independent esterification of the phosphate groups of DNA.

While it is now certain that cross-linkage under the alkylating agents cannot be the reason for their mutagenicity, yet it was thought feasible that this phenomenon might somehow influence the molecular basis of the induced mutations. Accordingly a detailed comparative study of the mutagenic properties of a series

of mono- and difunctional mesyloxy esters was undertaken. The point mutation visibles were analysed for mosaicism as opposed to complete expression. They were also compared with the lethals, both as regards frequency of induction and distribution along the chromosome. The frequency and types of the major chromosome rearrangements were also investigated. Special attention was given to the study of the physical nature of the recessive lethals as observed in the salivary gland chromosomes. The evidence from the various lines of inquiry pointed clearly to decisive differences in the mechanism of mutagenesis as a function of the number of alkylating groups per molecule.

MATERIAL AND TECHNIQUE

The compounds selected as representatives of the monofunctional mesyloxy esters R-OSO_2CH_3 are the methyl (CB.1540, R = CH_3) and the ethyl (CB.1528, R = C_2H_5) derivatives. The difunctional compounds belong to the dimethanesulphonylalkanols ($CH_3SO_2OCH_2$-$(CHOH)_n$-$CH_2OSO_2CH_3$): the mannitol (CB. 2511 and 2628, n = 4) and the erythritol (CB.2562, n = 2) derivatives. This selection is mainly due to the fact that these compounds offer no technical difficulties as regards administration by micro-injection in aqueous solutions, since they are all readily soluble in isotonic saline. Furthermore the mannitol disulphonate and the ethyl monosulphonate (at moderate doses) are virtually nontoxic to the injected flies, which enables the achievement of high mutagenicity at nonlethal doses.

Our micro-injection technique has been described in previous contributions (especially, FAHMY and FAHMY 1960b). The stock used is the wild type Oregon-K which gives in our laboratory a spontaneous sex-linked recessive mutation rate of 0.18 ± 0.05 percent lethals and 0.009 percent visibles (FAHMY and FAHMY 1959). The progeny of the treated males was fractionated according to our standard brood technique by their repeated mating to virgin females at three-day intervals, and the mutation rate was determined separately in each brood (FAHMY and FAHMY 1954, 1955).

The mutations analysed were: the autosomal dominants, the sex-linked recessive visibles, and the chromosome fragments and translocations, all from the progeny of attached-X experiments; also the sex-linked recessive visibles and lethals detected in the F_2 cultures from Muller-5 tests. The details of these techniques and our method of scoring have already been published (FAHMY and FAHMY 1955, 1956). Whenever possible, the mutants detected were confirmed in the F_3 and subsequent generations, and the visibles were subjected to allelism tests.

Samples of the sex-linked recessive lethals and the allelically unidentified visibles were genetically placed against a five-marker chromosome (scute, *sc*; cut, *ct*; vermilion, *v*; forked, *f*; carnation, *car*). A minimum of 300 males were scored in the crossover experiments. A sample of the sex-linked recessive lethals was examined cytologically in the salivary gland chromosomes. The preparations were aceto-orcein squashes, sometimes restained in Feulgen.

OBSERVATIONS

Mosaicism: The study of this phenomenon was undertaken on the visible mutants detected in the F_1 progeny from attached-X experiments. Care was taken to apply the same breeding procedure in tests with the different compounds. In particular, the progeny was collected within the same period after the insemination of the females, so as to standardize any possible effects of sperm storage in the spermathecae. The patroclinous males from treated fathers were carefully scored for any morphological abnormalities, and the aberrant flies were subjected to further genetic tests. A large proportion of the anomalous F_1 males could not be analysed genetically (Table 1), either because of sterility, or due to the lack

TABLE 1

The patroclinous sons obtained in attached-X experiments with males treated by various mesyloxy esters

Compound	CB.1540		CB.1528		CB.2511	
Dose ($\times 10^{-2}$M)	0.45		2.42		8.00	
	No.	Percent	No.	Percent	No.	Percent
Males observed	2979		7743		3966	
Phenotypically abnormal	333	11.18	1210	15.63	144	3.63
Died before test	17	0.57	4	0.05	—	—
Sterile	114	3.83	233	3.00	40	1.01
F_2 wild type	180	6.04	761	9.83	72	1.82
F_2 visible (complete or mosaic)	22	0.74	181	2.34	30	0.76
F_2 recessive lethal (no sons)	—	—	31	0.40	2	0.05

of recurrence of the mutant in the F_2, presumably due to gonadic mosaicism and nonheritable variations. It is to be noted, however, that the proportion of the unanalysable aberrant flies is not grossly different for the various compounds studied. The unanalysable sample, therefore, is unlikely to have introduced a major bias in the comparison between compounds.

The F_1 recessive visible mutants behaved differently on subsequent breeding (Table 2). In the analysis detailed in Tables 3–5, all the mosaic effects (Table 2, b–h) have been pooled. A very striking difference is immediately discernible as regards the degree of mosaicism between the monofunctional compounds and the difunctional representative (compare CB.1528, 1540 with CB.2511, Tables 2–5). The ratio of complete to mosaic mutations for the monofunctional agents is 1:2; whereas for the difunctional compound it is 2:1. A comparison of the data in this respect under the two monosulphonates with those for the mannitol disulphonate in a fourfold table revealed a significant difference ($x^2 = 6.24$ for one degree of freedom, $P \simeq 0.01$).

The F_2 X-visibles entered in Tables 3–5 were those detected by the Muller-5 method, and which were complete in both the soma and the germ line (breeding true in the F_3 and all subsequent generations). Nevertheless, their rate with all compounds was substantially higher than the combined complete and mosaic

TABLE 2

Progeny analysis of the F_1 sex-linked recessive visibles induced by various mesyloxy esters. Cv, complete visible; Mv, mosaic visible; s and d indicate same and different mutants respectively in the F_2 compared with F_1

Compound	CB.1540	CB.1528	CB.2511
Mutants tested:	10	142	15
Type: $F_1 \longrightarrow F_2$			
a) Cv Cs	3	49	10
b) Cv Ms	—	9	1
c) Mv Cs	4	45	3
d) Mv Ms	—	14	—
e) Cv Cd	2	8	—
f) Cv Md	—	3	—
g) Mv Cd	1	11	—
h) Mv Md	—	3	1
Ratio a/b-h	0.43	0.53	2.00

rates that could be established from the F_1 of attached-X experiments. This is a clear demonstration of the superiority of the Muller-5 technique in the detection of visibles. Many of the mutants which affect viability or fertility could be lost when isolated in the hemizygous patroclinous F_1 males. On the other hand, these would be transmitted through the heterozygous F_1 Muller-5 females and could subsequently be represented and detected among their F_2 sons.

The autosomal dominants detected in the present experiments are too few to enable any decisive conclusions. Nevertheless, it is sufficiently clear that with these mutations, as with the recessives, the ratio of the complete to mosaic mutants in the F_1 is higher for the difunctional compound (CB.2511, Table 5) than for either of the monofunctional derivatives (CB.1540, Table 3, CB.1528, Table 4).

The visible to lethal ratio: It has already been demonstrated that the ratio of visibles to lethals induced in the same sample of treated chromosomes could be a sensitive indicator of mutagenic differences between chemically related compounds (FAHMY and FAHMY 1960a). Accordingly, this property has been analysed in detail in the present study with the mesyloxy esters. The effect of the cell stage on this ratio was tested statistically for each compound by the contingency table technique, through a comparison of the frequency of the two mutant types in the separate broods from the same fractionation experiment. The data analysed in this way are detailed in Tables 3–5, and there was no indication of heterogeneity with any of the compounds: CB.1540, $\chi^2 = 3.6$, for four degrees of freedom, $P > 0.3$; CB.1528, $\chi^2 = 6.6$ for four degrees of freedom, $P > 0.1$; CB.2511, $\chi^2 = 2.2$, for four degrees of freedom, $P = 0.7$. It is clear, therefore, that under the mesyloxy esters the ratio of visibles to lethals is independent of the cell stage in which the mutants are induced. It should be pointed out in this connection, that with the sulphonates no disturbances in the visible to lethal ratio are likely to occur due to germinal selection. These compounds are only effective on the postmeiotic stages of the male germ line (FAHMY and FAHMY 1957, 1961), where no germinal selection is known to occur.

TABLE 3

Analysis of the mutations induced by 0.45 × 10^{-2}M of methyl methanesulphonate (CB.1540) in the F_1 of attached-X, and F_2 of Muller-5 experiments

Mutations \ Broods	I			II			III			IV			V			Total		
	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent
F_2 X-recessive lethals	455	53	11.6	506	53	10.5	677	59	8.7	482	44	9.1	494	12	2.4	2614	221	8.5
F_2 X-recessive visibles	455	21	4.6	506	12	2.4	677	15	2.2	482	9	1.9	494	2	0.4	2614	59	2.26
F_1 X-recessive visibles complete	448	1	0.22	1177	1	0.08	973	—	—	81	1	1.23	300	—	—	2979	3	0.10
F_1 X-recessive visibles mosaic	448	1	0.22	1177	3	0.25	973	3	0.31	81	—	—	300	—	—	2979	7	0.23
F_1 autosomal dominants complete	448	2	0.45	1177	4	0.34	973	2	0.21	81	—	—	300	3	1.00	2979	11	0.37
F_1 autosomal dominants mosaic	448	1	0.22	1177	—	—	973	—	—	81	—	—	300	—	—	2979	1	0.03

TABLE 4

Analysis of the mutations induced by 2.42 × 10^{-2}M of ethyl methanesulphonate (CB.1528) in the F_1 of attached-X, and F_2 of Muller-5 experiments

Mutations \ Broods	I			II			III			IV			V			Total		
	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent
F_2 X-recessive lethals	516	63	12.2	547	56	10.2	537	47	8.8	537	64	11.9	537	8	1.5	2674	238	8.9
F_2 X-recessive visibles	516	33	6.40	547	23	4.20	537	20	3.72	537	14	2.61	537	5	0.93	2674	95	3.55
F_1 X-recessive visibles complete	2126	18	0.85	1716	12	0.70	1271	9	0.71	952	7	0.74	1678	3	0.18	7743	49	0.63
F_1 X-recessive visibles mosaic	2126	31	1.46	1716	27	1.57	1271	24	1.89	952	10	1.05	1678	1	0.06	7743	93	1.20
F_1 autosomal dominants complete	2126	7	0.33	1716	10	0.58	1271	1	0.08	952	2	0.21	1678	—	—	7743	20	0.26
F_1 autosomal dominants mosaic	2126	10	0.47	1716	7	0.41	1271	1	0.08	952	1	0.11	1678	—	—	7743	19	0.25

TABLE 5

Analysis of the mutations induced by 8 × 10^{-2}M of dimethanesulphonyl mannitol (CB.2511) in the F_1 of attached-X, and F_2 of Muller-5 experiments

Mutations \ Broods	I			II			III			IV			V			Total		
	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent	Tested	Mut.	Percent
F_2 X-recessive lethals	531	29	5.5	533	20	3.8	531	26	4.9	531	44	8.3	508	11	2.2	2634	130	4.9
F_2 X-recessive visibles	531	5	0.94	533	2	0.38	531	6	1.13	531	9	1.69	508	4	0.79	2634	26	0.99
F_1 X-recessive visibles complete	638	3	0.47	948	3	0.32	722	2	0.28	1099	2	0.18	559	—	—	3966	10	0.25
F_1 X-recessive visibles mosaic	638	2	0.31	948	—	—	722	1	0.14	1099	—	—	559	2	0.36	3966	5	0.13
F_1 autosomal dominants complete	638	6	0.94	948	1	0.11	722	2	0.28	1099	4	0.36	559	1	0.18	3966	14	0.35
F_1 autosomal dominants mosaic	638	—	—	948	1	0.11	722	—	—	1099	—	—	559	—	—	3966	1	0.03

The effect of dose on the over-all visible to lethal ratio recovered from various fractions of the male germ line is given in Table 6. Particularly with the ethyl

TABLE 6

Ratio of sex-linked recessive visibles to lethals induced by various mesyloxy esters at different doses and various cell stages

Experiment No.	Dose ($\times 10^{-3}$M)	Days after treatment	Lethal rate percent	Chrom. tested	Visibles v	Lethals l	Ratio v/l
Alkyl monosulphonates							
CB.1528							
1.	1.00	0–3	6.1	489	11	30	0.37
2.	1.61	0–21	9.4	2481	63	234	0.27
3.	2.42	0–21	6.6	3762	97	250	0.39
4.	4.50	0–3	39.0	264	32	103	0.31
5.	9.18	0–15	13.8	355	10	49	0.20
Total				7351	213	666	0.32
CB.1540							
1.	0.25	0–21	3.3	3541	25	117	0.21
2.	0.45	0–21	6.6	3433	60	228	0.26
Total				6974	85	345	0.25
Polyol disulphonates							
CB.2511							
1.	1.00	0–21	1.0	3777	9	36	0.25
2.	4.00	0–3	2.3	479	4	11	0.36
3.	8.00	0–18	4.1	3700	32	150	0.21
4.	12.00	0–3	7.7	570	11	44	0.25
5.	16.00	0–21	7.2	1825	22	131	0.17
Total				10351	78	372	0.21
CB.2628							
	16.00	0–30	3.0	4822	34	146	0.23
CB.2562							
	16.00	0–21	1.2	3667	9	43	0.21

ester (CB.1528) among the monosulphonates and the mannitol derivative (CB. 2511) among the disulphonates, the experiments differed considerably both as regards the strength of the treatment, and the fraction of the germ line tested; yet no difference in the over-all visible to lethal ratio was detectable: CB.1528, $\chi^2 = 6.0$ for four degrees of freedom, $P = 0.2$; CB.2511, $\chi^2 = 2.3$ for four degrees of freedom, $P = 0.7$. The ratio of visibles to lethals under the mesyloxy esters, therefore, is independent of the strength of the treatment, as is the case with other alkylating mutagens (FAHMY and FAHMY 1955, 1960a).

Having established that the visible to lethal ratio is constant and characteristic for each compound of the mesyloxy series, a comparison was undertaken between chemical subseries on the basis of this property. It has already been shown

(FAHMY and FAHMY 1956, Table 3) that the dimethanesulphonoxyalkanes give a consistent visible to lethal ratio of 1:10. The polyol derivatives (CB.2511, 2628, 2562, Table 6), on the other hand, give a consistent ratio of 2:10 ($\chi^2 = 0.2$ for two degrees of freedom, $P = 0.9$). When the over-all data for these two subseries of the disulphonates were compared in a fourfold table, a significant difference was revealed ($\chi^2 = 7.7$ for one degree of freedom, ($P < 0.01$). The data for the monosulphonates are sufficient for a satisfactory determination of the visible to lethal ratio only in the case of the ethyl ester (CB.1528, Table 6) which gave a homogeneous ratio of 3 visibles:10 lethals. With the other monosulphonate (the methyl ester: CB.1540, Table 6) two experiments were undertaken, and one gave an anomalously low value (2:10, as with the polyol derivatives). Nevertheless, the over-all ratio for the methyl ester was still not significantly lower than the over-all value for the ethyl derivative ($\chi^2 = 3.3$, for one degree of freedom, $P > 0.05$). In contrast, a comparable test on the over-all relative frequency of the two mutant types under the ethyl (CB.1528) and the mannitol (CB.2511) derivatives revealed a very significant difference ($\chi^2 = 8.3$ for one degree of freedom, $P < 0.01$). It can safely be concluded, therefore, that among the mesyloxy esters the monofunctional compounds are more effective in the induction of visibles relative to lethals than the difunctional derivatives. The difference in this efficiency was less marked when the difunctional compound was a polyol derivative, thus showing that the structure of the prosthetic moiety may also be significant.

The genetical distribution of the lethals and visibles along the X chromosome under the mono- and disulphonates is illustrated in Table 7. To guard against

TABLE 7

Distribution of lethal and visible mutations among the five marked segments of the X chromosome under the mono- and difunctional mesyloxy esters

Chromosome segment	Lethals			Visibles		
	Mono-esters	Diesters	Total	Mono-esters	Diesters	Total
sc						
0–19.9	26	37	63	117	16	133
ct						
20–32.9	12	20	32	43	6	49
v						
33.0–56.6	22	40	62	106	20	126
f						
56.7–62.4	5	11	16	22	7	29
car						
62.5 +	4	14	18	10	2	12
Total	69	122	191	298	51	349

errors of placing in the linkage experiments pooling was undertaken in the five chromosome segments between the genetical markers. The mono- and difunctional compounds gave the same distribution as regards the lethals ($\chi^2 = 2.4$ for

four degrees of freedom, $P > 0.5$), as well as for the visibles ($\chi^2 = 3.2$ for four degrees of freedom, $P > 0.5$). The relative distribution of the lethals induced by all compounds was then compared to that of the over-all visibles and revealed a significant difference ($\chi^2 = 9.9$ for four degrees of freedom, $P < 0.05$). The partition of this heterogeneity χ^2, revealed that the significance is entirely due to the differential response of the proximal segment (*car*–centromere) which contributed to the total χ^2 a value of 8.0 for one degree of freedom ($P < 0.01$). It is, therefore, certain that the proximal part of the X chromosome yields a differential excess of lethals relative to visibles under the mesyloxy esters. This is a parallel situation to that already established for the phenylalanine mustard (FAHMY and FAHMY 1960a) and is subject to the same interpretation. The proximal part of the X chromosome, being predominantly heterochromatic, is apparently more vulnerable to mutation by material loss (gene elimination) which would favour the induction of lethals.

Chromosome aberrations: A comparison of the genetically detectable major chromosome rearrangements induced by three mesyloxy esters is given in Table 8. The data are particularly significant as regards the induction of viable

TABLE 8

The genetically detectable major chromosome rearrangements under various mesyloxy esters

Compound	X-recessive lethals			X-fragments			Translocations		
	Chr. tested	Lethals	Percent	Females observed	Fragments	Percent	Chr. tested	Trans.	Percent
CB.1528	2674	238	8.9	9192	—	—	2136	1	0.05
CB.1540	2614	221	8.5	3855	—	—	1500	—	—
CB.2511	2634	130	4.9	4300	7	0.16	2026	—	—

fragments. The difunctional mannitol derivative (CB.2511) was effective in the production of these aberrations, whereas both monofunctional compounds (CB. 1528, 1540) were completely ineffective in this respect at double the mutagenic dose (as regards lethal rate). Even when the difference in the lethal rate was ignored, the absence of the fragments in the experiments with the monofunctional compounds still reached statistical significance (CB.1528: $\chi^2 = 15.0$ for one degree of freedom. $P < 0.001$; CB.1540: $\chi^2 = 6.3$ for one degree of freedom, $P < 0.02$). A more accurate statistical comparison was also undertaken allowing for the higher mutagenic dose under the monofunctional compounds, assuming that the rate of fragments increases as (lethal rate)$^{\frac{9}{2}}$. On the basis of the observed rate of fragments under the mannitol disulphonate (CB.2511, Table 8), the experiments conducted with the ethyl and methyl monosulphonates (CB.1528 and 1540, Table 8) would be expected to yield 35.8 and 13.9 fragments respectively. Instead, both compounds induced no fragments ($P < 0.001$ for either agent), thus indicating the very gross lower efficiency of monofunctional compounds in the induction of chromosome breaks. In contrast, the rate of recoverable fragments under the mannitol disulphonate approaches that occur-

ring under X-radiation. An experiment with X-rays conducted with the same size sample and at the same mutagenic level as the mannitol compound (CB.2511, Table 8) would be expected to give 11.6 recoverable X chromosome fragments, which is not significantly different from the seven observed with the chemical agent ($\chi^2 = 1.1$, for one degree of freedom, $P \simeq 0.3$).

The data as regards the induction of translocations are included in Table 8 and show that the sulphonates, whether mono- or difunctional, are almost ineffective in the induction of these rearrangements. It is of interest to note, however, that the single translocation detected occurred with the monofunctional compound ethyl methanesulphonate. While it is clear that this does not constitute a significant difference from the difunctional compound, yet it shows that even monofunctional agents may, very infrequently, break the chromosome.

The chromosome aberrations induced among the sex-linked recessive lethals and observed in the salivary gland chromosomes are illustrated in Table 9. With

TABLE 9

Cytological analysis of the sex-linked recessive lethals induced by a mono- and a dimesyloxy ester

Compound	Stocks	Lethals percent	Major rearrangements					Deficiencies	
			Del.	Inv.	Trans.	Total	Percent	No.	Percent
CB.2511	94	15.2	4	4	3	11	11.7	39	41.5
CB.1540	83	11.2	1	—	—	1	1.2	14	16.9

both the mono- and difunctional compounds the mutants came from the first four broods (0–12 days after treatment) and must have been induced in postmeiotic germ cells (sperm and spermatids). In view of the difference in the mutation rate in the different broods, the over-all mutagenic level was estimated as the weighted mean rate in the broods from which the observed lethals were selected; the weight was taken as the number of lethals observed per brood.

The data in Table 9 clearly show that the potency of the mannitol disulphonate is significantly higher than that of the methyl monosulphonate both as regards the induction of major rearrangements ($\chi^2 = 7.7$, for one degree of freedom, $P < 0.01$) and small deficiencies ($\chi^2 = 8.7$, for one degree of freedom, $P < 0.01$). In view of the higher mutagenic dose (as regards the lethal rate) under the difunctional agent, it was thought appropriate to adjust the observed chromosome aberration rates under the monofunctional compound. This was raised according to $(\text{lethal rate})^{\frac{3}{2}}$ for the rearrangements, and in direct proportion to this rate for the deficiencies. The expected frequencies thus calculated for the monofunctional compound were then compared to those induced by the difunctional agent. This comparison still revealed a significant excess of chromosome damage under the mannitol disulphonate, both as regards major rearrangements ($\chi^2 = 4.4$, for one degree of freedom, $P < 0.05$) and small deficiencies ($\chi^2 = 6.3$, for one degree of freedom, $P \simeq 0.01$).

DISCUSSION

Several important differences were established in the mutagenic properties of the mesyloxy esters as a function of the number of active groups per molecule. The difunctional derivatives were more effective than the monofunctional compounds in the induction of:

1. Complete relative to mosaic visible mutants.
2. Lethal relative to visible recessive mutants.
3. Chromosome breaks leading to major rearrangements.
4. Gene inactivation resulting in small deficiencies.

On the molecular level, all the above differences are reconcilable with a larger "target of damage" under the difunctional agents. This could conceivably be the consequence of intra- and intermolecular cross-linkage of the genic DNA, in a manner akin to that which has been demonstrated *in vitro* (ALEXANDER, COUSENS and STACEY 1957). A few cross-linkage reactions per DNA molecule were shown to alter the physical nature of the macromolecule, to a degree which is bound to result in its biological inactivation. Monofunctional compounds, on the other hand, could only produce "blockages" of isolated DNA groups, and could accordingly induce in the macromolecule much smaller damage: of submolecular dimensions. These small sites of damage, however, must be sufficient to alter the transmission of the genetic code since they do result in mutations.

The higher relative rate of induction of complete to mosaic visible mutants under the difunctional compounds could be attributed to cross-linkage across the chromosome. This immediately brings into the picture, the *in vitro* demonstration (ALEXANDER *et al.* 1957) that difunctional agents could attack and break both spirals of the DNA molecule, whereas the monofunctional compounds can break only one of the twin chains of the dimeric structure. While it is certain that gene structure and organization is infinitely more complex than DNA *in vitro*, yet it is becoming gradually clear that genic activity primarily resides in DNA, and gene structure fundamentally conforms to the Watson-Crick model. It would seem reasonable, therefore, to associate a higher incidence of the twin chain type of reaction (cross-linkage) within the genic DNA, with a higher relative frequency of complete mutants—both being favoured by difunctionality. Doubtless, however, mosaicism involves secondary processes (on the chromosome level) subsequent to the initial molecular damage. On the basis that the chromosome consists (at the moment of damage) of a string of genes with twin DNA strands, the minimum damage could only involve a single strand. This would result in half-mutant F_1 flies, when in fact mosaic individuals were observed with only small areas showing the mutation. Two hypotheses have already been suggested for the explanation of these mosaics (BIRD and FAHMY 1953): (a) partial chromosome damage, or (b) delayed action; and both can now be amplified to accomodate a molecular interpretation. Small area mosaics could be interpreted on the partial damage hypothesis, on the basis that the treated chromosome was more than one DNA molecule across at the time of treatment. The delayed action hypothesis could also explain extreme mosaics, if it can be assumed that single

strand DNA breaks could restitute during embryonic development, allowing "genic recovery" in some tissue anlage. It should here be noted, however, that the assumed delay in the present interpretation is entirely in the process of recovery and not that of damage as postulated in the original hypothesis.

Evidence has been adduced that recessive lethals are mutations involving some loss of the genetic material, whereas visibles are mainly the outcome of intramolecular reorganization within genes (FAHMY and FAHMY 1960a). The higher efficiency of difunctional agents in the induction of lethals relative to visibles may well be attributed to their greater potentiality for gene inactivation. More significant in this connection is the fact that the lethals of the difunctional compounds were associated with a higher frequency of cytologically detectable deficiencies. This higher efficiency for gene inactivation under the difunctional agents can most plausibly be the outcome of intermolecular cross-linkage of the genic DNA along the chromosome.

The difunctional mesyloxy ester is far more effective than the monofunctional derivatives in the induction of chromosome breaks leading to major rearrangements, especially chromosome fragments. This strongly suggests that cross-linkage is perhaps more relevant to chromosome breakage, rather than point mutations. It is nevertheless certain that chromosome breaks could be produced by monofunctional compounds, though most infrequently (Tables 8 and 9). The mechanism here involved is doubtless independent of cross-linkage, but could be due to the instability of the polynucleotide chain of alkylated DNA. The phosphate triesters produced are easily hydrolysed, usually with the splitting off of the alkylating agent, but occasionally the sugar-ester bond is broken resulting in the interruption of one of the DNA chains (ALEXANDER *et al.* 1957). A similar fission of one of the DNA chains could also ensue by hydrolysis, if the initial alkylation occurs on the tertiary ring nitrogen of guanine moieties (LAWLEY 1957). At high mutagenic levels (excess alklylation), it is conceivable that two nearby single chain breaks might occur in the genic DNA, thus resulting in a full break across the whole molecule. This could obviously constitute a mechanism for the initiation of chromosome breaks, though it would naturally be less effective than cross-linkage under the difunctional compounds.

SUMMARY

The nature of the mutants induced by the mesyloxy esters varied according to whether the molecule was mono- or difunctional. The presence of two active groups (the mesyloxy radicals) favoured the induction of: (a) complete relative to mosaic mutants, (b) a high lethal to visible ratio, (c) more chromosome breaks and (d) a higher frequency of small deficiency recessive lethals. These observations indicated a larger target of action on the part of difunctional agents, as might occur as a consequence of intra- and intermolecular cross-linkage of the genic subunits.

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